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ENGRAFTMENT AND TOLERANCE INDUCTION IN CELLULAR THERAPY
- FOCUS ON MESENCHYMAL STROMAL CELLS

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Till Anders, Folke och Ebbe

Nog finns det mål och mening i vår färd -
men det är vägen, som är mödan värd

Karin Boye

Engraftment and tolerance induction in cellular therapy - Focus on mesenchymal stromal cells

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Different cell types have been employed in the search for a cellular therapy that is applicable for the treatment of heart failure. Remuscularization strategies using stem-cell-derived cardiomyocytes have been prone to arrhythmias. Mesenchymal stromal cells (MSCs) are one of the most promising cell types for the treatment of heart failure due to their diverse regenerative properties. We have isolated MSCs from the human fetal heart in the form of human fetal cardiac MSCs (hfcMSCs), which might comprise a more relevant cell source than bone marrow-derived MSCs, for the treatment of heart failure. Although the cell source is of importance for successful cell therapy, the efficacy of the cells also depends on avoiding anchorage-dependent apoptosis (anoikis) and immune rejection. Immune rejection may be counteracted with immunosuppressive drugs; however, these drugs generally have serious side effects that limit their suitability in cell therapy. We have used a transient blockade of T cell costimulation with the aim to induce immunologic tolerance through the generation of Regulatory T cells (Tregs) specifically for the antigens of the transplant. Anoikis may be diminished by the simultaneous administration of the exogenous extracellular matrix (ECM), which provides anchorage possibilities for the transplanted cells.

In Paper I and II the use of costimulation blockade in xenogeneic and allogeneic mouse models was explored and we showed that $Foxp3^+$ Tregs are associated with tolerated and viable grafts as well as reduced reactivity in mixed lymphocyte reaction tests. MSCs, known for their immunomodulatory properties, were shown to synergize with costimulation blockade to facilitate immunological tolerance to allogeneic insulin-producing islets in mice.

In Paper III we showed that Matrigel synergizes with costimulation blockade to enable enhanced cell retention of hfcMSCs after subcutaneous implantation in mice. The hfcMSCs displayed vasculogenic potential *in vivo*, shown as the expression of CD31, and also the formation of vessel-like structures staining positive for laminin α4. In Paper IV, hfcMSCs were cultured on a 3D scaffold composed of a novel type of artificial spider silk, NT2RepCT, to conserve the cell connections and ECM at the time of implantation. The hfcMSCs displayed vasculogenic potential in the 3D cultures on NT2RepCT as well, with the formation of an ECM that stained positive for laminin α4 and fibronectin and contained vessel-like structures expressing CD31. After *in vivo* implantation, the hfcMSCs on the NT2RepCT were subjected to immune rejection regardless of costimulation blockade or isotype control treatment.

In this thesis hfcMSCs were shown to have a vasculogenic potential as they also deposit laminins that are important for vascular structures and thereby may have the potential to prevent ischemic heart failure. We also demonstrated a synergistic effect between costimulation blockade and MSCs or Matrigel for enhanced cell retention. Artificial spider silk showed great potential for advanced *in vitro* studies with 3D cultures, but could not be used *in vivo* in its current form.

LIST OF SCIENTIFIC PAPERS

I. Costimulation Blockade Induces FoxP3+ Regulatory T Cells to Human Embryonic Stem Cells
Karin Ljung, Oscar E. Simonson, Ulrika Felldin, Ewa Wärdell, Cristian Ibarra, Liselotte Antonsson, Makiko Kumagai-Braesch, Outi Hovatta, Riina Lampela, Karl-Henrik Grinnemo, and Matthias Corbascio
Bioresearch Open Access. 2013; 2(6):455-458

II. Multipotent Mesenchymal Stromal Cells Synergize with Costimulation Blockade in the Inhibition of the Immune Responses and the Induction of FoxP3+ Regulatory T Cells
Tohru Takahashi, Annika Tibell, **Karin Ljung**, Yu Saito, Anna Grönlund, Cecilia Österholm, Jan Holgersson, Torbjörn Lundgren, Bo-Göran Ericzon, Matthias Corbacio, Makiko Kumagai-Braesch
Stem Cells Translational Medicine 2014; 3(12): 1484-1494

III. Human Fetal Cardiac Mesenchymal Stromal Cells Differentiate in vivo into Endothelial Cells and Contribute to Vaculogenesis in Immunologically Competent Mice
Karin Ljung, Anna Grönlund, Ulrika Felldin, Sergey Rodin, Matthias Corbascio, Cecilia Österholm*, Karl-Henrik Grinnemo*
Submitted to Stem Cells and Development

IV. Artificial Spider Silk Provides a 3D Scaffold for the Growth, Vasculogenesis and Matrix Formation of Human Fetal Cardiac Mesenchymal Stromal Cells
Karin Ljung, Marlene Andersson, Lotta Floderus, Kerstin Nordling, Matthias Corbascio, Jan Johansson, Cecilia Österholm, Karl-Henrik Grinnemo,* Anna Rising*
Manuscript

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LIST OF ABBREVIATIONS

APC	Antigen-presenting cell
ARDS	Acute respiratory distress syndrome
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
FISH	Fluorescent in situ hybridization
Foxp3	Forkhead box 3
GvHD	Graft-versus-host disease
HESC	Human embryonic stem cell
hfcMSC	Human fetal cardiac mesenchymal stromal cell
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule 1
IDO	Indoleamine 2,3-Dioxygenase
IFNγ	Interferon γ
IL-2	Interleukin 2
IL-10	Interleukin 10
iPSC	Induced pluripotent stem cell
LFA-1	Lymphocyte function-associated antigen 1
MSC	Mesenchymal stromal cell
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
NK cell	Natural killer cell
NSG	Nod scid gamma
PGE2	Prostaglandin E2
TGFβ	Transforming Growth Factor β
TNFα	Tumor Necrosis Factor α
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

The loss of functional tissue due to cell death is a major component in the pathogenesis of several diseases. Offering a cure in the form of cell therapy, where the missing cell population is transplanted to the patient, has been, and remains, a developing area of research. Patients with diabetes mellitus type 1, a condition in which insulin-producing islet cells are destroyed, can receive new cells that are usually delivered through a catheter to the superior mesenteric vein[1] – a kind of cell therapy. Heart disease is another area where cell therapy is of great interest. Heart failure affects about 2% of the population in the developed world[2] and ischemic heart disease is a major cause of heart failure with reduced ejection fraction (EF)[3]. The abrupt loss of nutrients and oxygen when the blood supply is blocked during an ischemic event results in the remodeling of the myocardium, fibrosis and loss of function.[4] There are medical treatments and devices, like cardiac resynchronization therapy (CRT), that may improve heart function;[5] however, the prognosis has not improved in the last decade.[6] Heart transplantation is the only potential cure for heart failure, though organ shortages and the adverse effects of immune suppressants pose serious limitations to its application. This has resulted in a treatment that is available only to a small group of patients and accompanied by severe comorbidities, such as opportunistic infections and kidney failure.

In 2000, the first clinical trial of cell therapy for heart failure was performed using myoblasts.[7] Since then clinical trials with bone marrow cells,[8] mesenchymal stromal cells[9,10] and the cells of a cardiac progenitor profile[11,12] have been conducted with the aim to improve heart function and reduce mortality for heart failure patients. Cellular therapy could be an option between medical treatment and heart transplantation offering the patient something approaching a cure, but at the same time increasing the availability of the treatment and decreasing the risks. However, even if the results from clinical trials with bone-marrow-derived cells indicate low risk to the patient,[13] a meta-analysis from 2015 showed no convincing improvement in heart function.[14] These negative results might be explained by the poor cell retention observed in both animal models[15] and clinical trials,[16] which is well illustrated in a syngeneic rat model that failed to show any relationship between the number of delivered cells and the number of engrafted cells at four weeks.[17] Increasing cell survival after delivery is both important and difficult. Immune rejection and the loss of cell anchorage must be targeted and of course the appropriate cell source for a given condition has to be established.

1.1 CELL SOURCE

For the diabetic patient, it is evident that insulin-producing islets are lacking and the therapy should aim to replace them. In heart failure, the picture is more complex. Heart failure is often caused by ischemic heart disease,[3] wherein the ischemia has resulted in a tissue defect and scar

formation. Heart tissue consists of muscle cells, supportive cells (cardiac fibroblasts) and vessels, forming a complex and important connection for normal heart function. The cell type or combination of cells that offers the best potential and feasibility needs to be carefully considered.

1.1.1 Cardiomyocytes

The obvious choice for cell therapy aiming to restore heart function is the delivery of cardiac muscle cells. These cells may be derived from human embryonic stem cells (HESCs) or induced pluripotent stem cells (iPSCs). Smaller animal models, including mice,[18] rats[19] or guinea pigs,[20] have shown promising results with engrafted cardiomyocytes. However, when translated to non-human primates, ventricular arrhythmias were observed with HESC-derived cardiomyoctes[21,22] as well as iPSC-derived allogeneic cardiomyocytes.[23] In the aforementioned studies, xenogeneic or allogeneic cardiomyocytes coupled with the host cardiomyocytes, as visualized through a fluorescent calcium indicator (GCaMP2).[24] The cardiomyocytes produced from HESCs and iPSCs were immature and likely differed in their conduction properties from host cardiomyocytes. This was illustrated by their reduced expression of connexin 43,[25] which may comprise part of the explanation for the observed ventricular arrhythmias. Arrhythmogenesis is a major obstacle to any clinical application of cardiomyocyte-based cellular therapy.

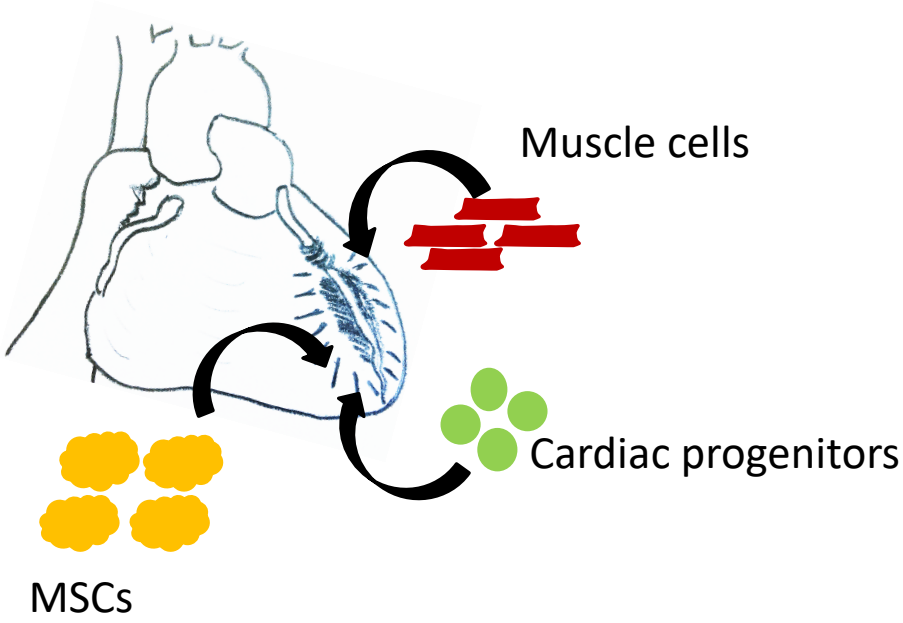


Figure 1. Cells of different origins have been utilized in the search for an effective cure for heart failure. Initially muscle cells, including skeletal myoblasts and cardiomyocytes (animal models), were used as replacement therapy. Cardiac progenitor cells have been hard to define and have been assumed to be more plastic. Mesenchymal stromal cell (MSC) therapy mainly aims to contribute to a local positive paracrine effect, enhancing the endogenous repair mechanisms.

1.1.2 Mesenchymal stromal cells

MSCs were initially isolated from the bone marrow by Alexander Friedenstein in 1970.[26] He found that when bone marrow cells were plated on a culture dish a portion of the cells would rapidly attach and these cells formed fibroblast-like cultures. Subsequently, these cells were found to form both bone and cartilage under certain conditions.[27] Human MSCs were first characterized by Pittinger et al. in 1999[28] and have been retrieved from many different locations, including the adipose tissue.[29] MSCs are considered to reside close to blood vessels and resemble pericytes,[30] the cells that encapsulate smaller blood vessels and capillaries. According to the International Society for Cellular Therapy, MSCs should adhere to plastic culture dishes, express CD105, CD73 and CD90, lack CD45, CD34, CD11b or CD14, CD79a or CD19 and Human Leukocyte Antigen (HLA) DR expression and be able to differentiate into bone, cartilage and fat.[31] MSCs are considered to exhibit immunomodulatory, pro-angiogenic and wound-healing properties.[32] The immune modulation is achieved through both soluble factors and cell–cell interaction. This has been shown in experiments using transwell systems,[33,34] and affects both the innate and the adaptive immune response. The immunomodulatory effect of MSCs is triggered by strong pro-inflammation through Tumor Necrosis Factor α (TNFα) and Interferon γ (IFNγ),[35] which are mainly released by T cells and macrophages as a response to inflammatory stimuli. MSCs may then release a range of molecules that are important in immune modulation, including Indoleamine 2,3-Dioxygenase (IDO), Prostaglandin E2 (PGE2), Interleukin 10 (IL10), Transforming Growth Factor β (TGFβ) and HLA-G5.[36]

Macrophages may be divided into two subsets, M1 and M2, wherein M1 macrophages are pro-inflammatory, producing IFNγ and TNFα, and M2 macrophages are anti-inflammatory, producing IL-10 and promoting wound-healing.[37] MSCs have been shown to skew the macrophages toward an M2 profile and PGE2 is essential for this function.[38] The MSCs are believed to have a modulatory effect on the adaptive immune response, largely due to the suppression of T cell proliferation[33,34] and the induction of regulatory T cells[39] (Tregs) that promote immune tolerance.[40] This process is performed in several steps, where MSCs first produce TGFβ,[36] which promotes Tregs' induction from naïve $CD4^+$ T cells.[41] Secondly, MSCs release IDO in response to high levels of IFNγ.[35] IDO modulates dendritic cells (DCs), which are specialized antigen presenting cells (APCs), towards a tolerogenic profile, that induces Tregs.[35,42] Thirdly, MSCs express HLA-G5, which induces Treg expansion.[43] HLA-G is an isoform of the non-classical HLA molecule (low degree of genetic variance) found in the trophoblasts. HLA-G is tightly connected to immune tolerance towards the fetus during pregnancy, where Tregs are involved, and low levels of HLA-G are connected to preeclampsia and miscarriages.[44] HLA-G5, along with PGE2 release, is also implicated in the suppressive effect of MSCs that is exerted on natural killer cells (NK cells),[37,43] which are innate lymphocytes with cytotoxic properties activated by a lack of MHC-class I molecules. The major role of NK cells is to respond to tumors and virus infections, but they are also involved in the rejection of transplants. Taken together, *in vitro* observations indicate that MSCs react to strong inflammatory stimuli by producing factors that interfere with the inflammatory chain

reaction, which may otherwise cause excessive tissue damage. In response to mild inflammatory stimuli, MSCs react differently. When subjected to weak inflammatory stimuli, MSCs produce antibacterial peptides[45] and promote inflammation mainly by recruiting and stimulating neutrophils.[36,37] This dynamic response may implicate MSCs as an important part of our innate immune system, granting that the *in vitro* data is applicable to the *in vivo* situation.

In 2000, MSCs were first used in a clinical trial.[46] Patients with stage IV metastatic breast cancer received autologous MSC infusions along with hematopoietic stem cells to facilitate engraftment after myeloablative therapy. No adverse event related to the MSCs was observed. Subsequently, when theories of MSCs' immunomodulatory potential have arisen, MSC-infusions have been used successfully to treat severe graft-versus-host disease[47,48] (GvHD) and acute respiratory distress syndrome[49] (ARDS). The therapeutic effects observed are considered to relate to the paracrine factors described before as well as to the release of exosomes.[50] GvHD and ARDS are conditions with misdirected overactive immunity, though the short-term immunomodulatory effect of MSCs seems to be enough to break the vicious inflammatory cycle, and cell engraftment or survival is not important in that context.

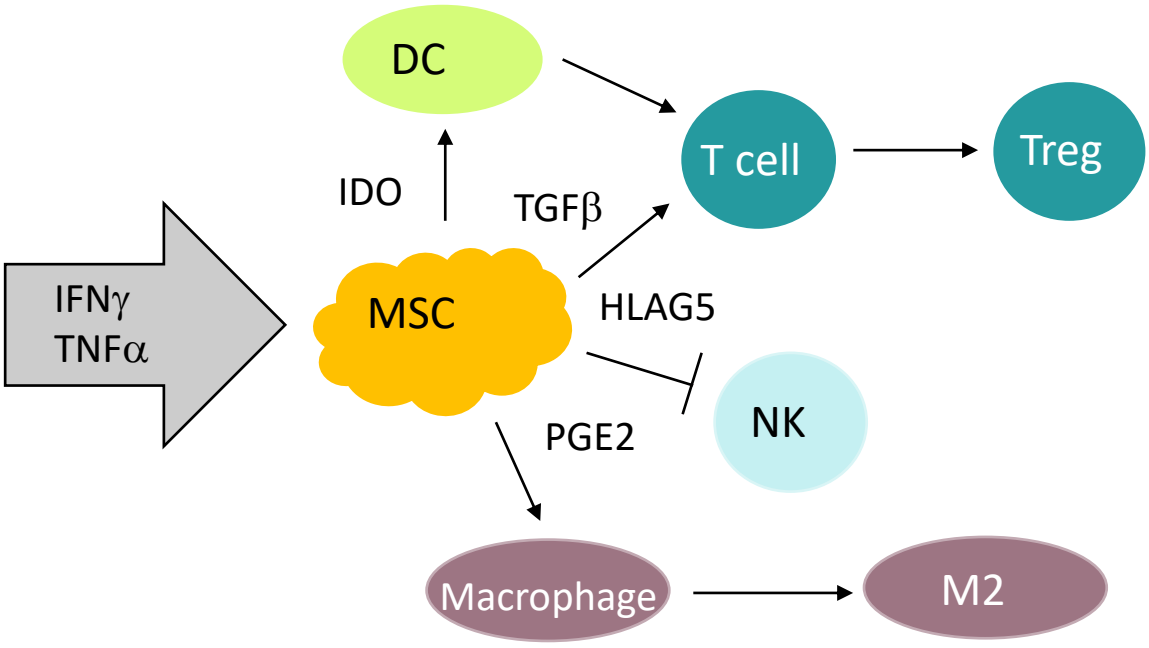


Figure 2. A simplified overview of the response of MSCs to strong inflammatory stimuli, resulting in tolerogenic DCs, Treg induction, NK cell inhibition and an anti-inflammatory M2 profile of macrophages.

MSCs are considered to have an almost exclusively paracrine function,[51] and actual cell contribution to the tissue is not expected. However, whereas an intense effect for a short time is optimal for extreme inflammatory states, like GvHD and ARDS, the pathogenesis of heart failure is different. In considering an ischemic heart muscle with scarring and remodeling, it is reasonable to assume that a prolonged exposure to paracrine factors with pro-angiogenic and immunomodulatory activities would be optimal. Therefore, it is important to increase MSCs' engraftment when considering them for the treatment of heart disease. It has been hard to evaluate MSC-engraftment in the human heart. Autopsy material from patients who received infusions of MSCs for various other conditions has shown a very low rate of engraftment.[52] Turning to animal models for a more precise answer yields conflicting results. Some studies show that MSCs survive long term after delivery to the heart without immune suppression, but in several of these experiments lipophilic dye or ferumoxide was used to label the cells.[53,54] These labels are not specific, since the dye is transferred to neighboring cells to a high extent[55] and the ferumoxide is accumulated in phagocytes, like macrophages,[56] making the results hard to interpret. When other more robust methods, like GFP labeling or fluorescent in situ hybridization (FISH), were used to detect cells, no or very few MSCs were retained in the tissue a few weeks after transplantation.[15,57,58]

1.1.3 Human fetal cardiac mesenchymal stromal cells

The heart develops early during embryogenesis and a beating heart can be observed already in gestational week six. MSCs can be retrieved from fetal tissue and have been isolated from the human fetal heart.[30,59] Fetal MSCs could be more advantageous to use in cell therapy due to their increased telomerase activity and increased proliferation rate.[60] Cells originating from a first-trimester fetal heart differ from adult sources since these cells have the capacity to generate the components of the heart, including the endothelial cells, smooth muscle cells and, to a minor extent, cardiomyocytes *in vitro*.[59] Due to their origin and potential to contribute to the different components of the heart, human fetal cardiac MSCs (hfcMSCs) may have a greater potential for cellular replacement therapy after a myocardial infarction.

1.2 ANOIKIS

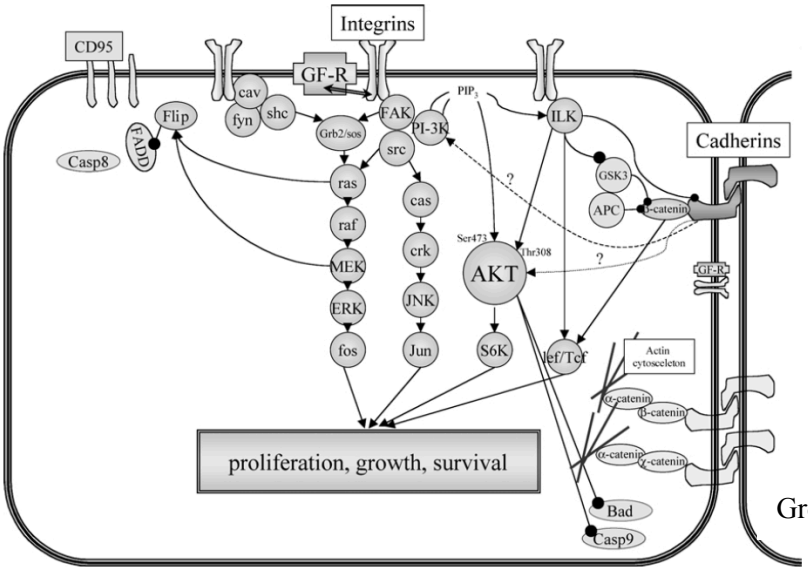
1.2.1 Integrins, cadherins and the extracellular matrix

As parts of a tissue, cells form bonds between themselves and neighboring cells as well as with the extracellular matrix (ECM). Many molecules participate in cell adhesion, with cadherins and integrins being of central importance. Cadherins mainly take part in cell–cell interaction[61] and integrins are involved in the formation of adhesive contacts between the ECM molecules and the cells.[62] Both are transmembrane molecules with close links to the actin cytoskeleton.[63] Ligands binding to cadherins and integrins initiate the cellular pathways involved in proliferation,[64] differentiation and migration and form complicated networks of interactions

between themselves that may be referred to as "adhesive crosstalk".[63] As this term indicates, cadherins and integrins participate in the communication between the cell and its surroundings. Cadherins also take part in the formation of the desmosome, which is connected to the intermediate filaments of the cell and confers the strong cell adhesions found in the skin and heart muscle.[65]

Collagens, glycoproteins like laminins and fibronectin, and proteoglycans make up the ECM, which is a dynamic structure distributed throughout the organism with functions ranging from the construction of the mechanical milieu of an organ to its status as the reservoir of growth factors.[66] Adhesion to the ECM is facilitated through the ligation of an ECM molecule to an integrin. The cellular response may enhance insulin release or angiogenesis, for example, depending on the ECM component, integrin and cell type involved.[67,68] The ECM's composition varies between organs and a lack of certain components may cause disease. For example, laminin α4 deficiency in mice has been shown to cause cardiomyopathy,[69] which is likely mediated through difficulty establishing contact with blood-vessels.

If connections between cadherins and integrins to other cells and the ECM molecules are missing, the cell will experience a loss of anchorage. In most cells, inactivated integrins or cadherins will fail to confer survival signals, stimulating apoptotic signaling instead.[70,71] The loss of anchorage to the ECM and other cells may also cause apoptosis through Fas and Fas ligand upregulation, as well as Fas relocation when the cell changes shape due to different surroundings.[71] The apoptosis, induced by the loss of anchorage, is usually referred to as anoikis and is considered to be a major reason for low cell survival and the loss of graft function in cell therapy.



Grossman et al, Apoptosis, 2002

Figure 3. Illustration of the complex pathways involved in integrin and cadherin signaling. Proliferation, growth and survival of the cell is stimulated and apoptotic pathways are blocked through anchorage to other cells and the ECM by the ligation of integrins and cadherins. If these anchorage signals are missing, the blockage of apoptotic pathways is instead removed, which may result in anoikis.

1.2.2 Strategies for cell delivery

Injection of a single cell suspension is the common way to attempt cell implantation to the heart. To achieve this, cells are loosened from the culture plate using an enzymatic reaction that strips off adhesion molecules and thereby reduces their linkage capacity to other cells and the ECM. After this treatment, the cells are passed through a thin needle, increasing the risk of apoptosis, probably due to mechanical effects.[72]

Insulin-producing islets meant for transplantation receive a similar treatment. They are infused through a thin catheter after retrieval from the pancreatic tissue, which is achieved through a collagenase treatment that digests the surrounding ECM.[73] This treatment results in an increased loss of anchorage to the ECM after the implantation of the islet cells, which might cause anoikis and contribute to early graft loss. A small study conducted on Rhesus monkeys indicated improved islet survival when part of the ECM was kept or the anti-apoptotic molecule Bcl-2 was supplied,[74] further supporting anoikis' involvement in graft loss. Other ways to directly reduce anoikis through the addition of ECM components and anti-apoptotic agents have been successful in the transplantation of cardiomyocytes into the hearts of rats and macaques.[19,21] The authors developed a pro-survival cocktail that included Matrigel, apoptosis inhibitor Bcl-XL and a caspase inhibitor. The work devoted to developing the techniques described above emphasizes the problem anoikis poses to any cell therapy in otherwise causing a low cell survival and engraftment. Among all the different methods developed to limit anoikis, one may identify two fundamentally different approaches: Cells are either mixed with ECM components before delivery or cells are delivered as a 3D structure, where cell–cell and cell–ECM interactions are conserved.

1.2.3 ECM components to reduce anoikis

The delivery of cells through a thin needle is technically easy and the anoikis induced by the single cell state,[70,71] where integrins and cadherins lose their potential for ligation, may be avoided through the addition of a hydrogel or Matrigel to the cell suspension.[19,75,76] Matrigel is frequently used for cell culture and has been shown to improve *in vivo* engraftment and the expression of integrins compared to PBS in a model of HESC-derived endothelial cells.[77] Matrigel consists of the ECM produced by chondrosarcoma cells, Engelbreth-Holm-Swarm (EHS), with a composition resembling that of basement membranes, including collagen IV and laminin 111, in addition to numerous growth factors.[78] The positive effect of Matrigel on engraftment is likely due to the presence of these basement membrane proteins to which cells may form integrin connections and thereby avoid anoikis. Through its gelling properties, Matrigel will also restrict cell dispersion, and thereby increase the probability of cell–cell connections through cadherins, further decreasing the risk of anoikis. Basement membranes may vary in composition but collagen IV, perlecan and/or agrin (heparan sulfate proteoglycans), laminins and nidogens are always present.[79]

Perlecan and other heparan sulfate proteoglycans sequester growth factors to the ECM, like TGFβ and vascular endothelial growth factor (VEGF),[79] which indicates that Matrigel, in

addition to inhibiting anoikis, serves as a reservoir for growth factors after its delivery to the tissue. Although there are many positive sides to Matrigel, its origin in mouse tumor cells and varying composition makes clinical use hard to imagine. In a clinical setting, hydrogels may be a better choice, but for *in vitro* and *in vivo* studies of animals where the importance of anoikis needs to be sorted out, Matrigel is still a valuable product.

1.2.4 3D scaffolds to reduce anoikis

An alternative to injecting or infusing cells could be the implantation of a 3D scaffold with cells attached. Implanting a 3D scaffold might be technically more advanced than injecting cells, but the advantages to engraftment could be immense, considering that the cell–cell and cell–ECM bonds that were established in the culture are conserved during implantation. Another advantage is the possible reduction of tissue damage and ischemia. Injection could cause ischemia by increasing the pressure in the tissue when cells are injected to the heart muscle. The infusion of insulin-producing islets, and possibly also other cells, causes graft-ischemia when they are trapped in the venules of the liver.[1] Ischemia attracts inflammatory cells, which may further increase the risk of apoptosis, necrosis and immune rejection. Scaffolds consisting of different hydrogels, fibrin or Matrigel have been studied in animal models with encouraging results.[80-82]

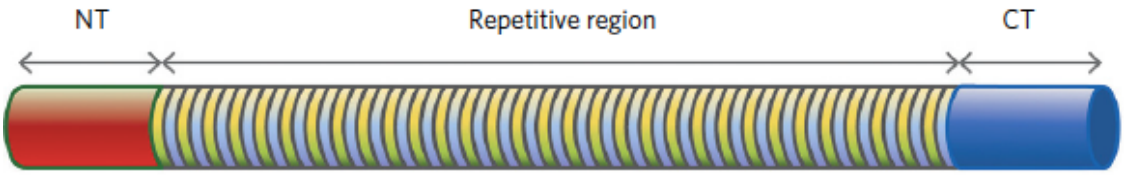
An alternative to scaffolds that mimic the ECM is the use of spider-silk-based scaffolds for 3D culture and implantation. Spider silk is a material with the unique mechanical properties of extreme strength and high flexibility.[83] It is composed of proteins with a mainly β-sheet structure, giving spider silk an amyloid-like configuration.[84] A 3D scaffold composed of spider silk fibers would aim to provide a skeleton to support the generation of a new ECM by the cultured cells. This differs fundamentally from the other scaffolds discussed that provide ECM components to the cells.

Originally, the most commonly used type of silk was derived from silkworms. Silkworm silk has been used by humans for centuries in clothing and later also in surgical sutures. However, native silkworm silk may cause allergic reactions and inflammation, probably due to the combination of sericin coating and fibroin core.[85] Another type of silk fiber is produced by spiders and is stronger with a higher extensibility and stiffness than the silk produced by silkworms.[83] However, spiders are territorial and therefore difficult to keep over time for the production of larger quantities of spider silk. To overcome this problem recombinant spider silk has been developed.

Natural spider silk proteins (spidroins) are composed of non-repetitive C- and N-terminal domains flanking a repetitive region. These spidroins are generally about 3,000 to 4,000 amino acids residues long and are hard to efficiently express in bacteria.[86] Stark et al. developed plasmids to express smaller units of the spidroin, which are still able to form silk-like structures in test tubes that are slowly tilted from side to side. According to their concept, one C-terminal domain (CT) is connected to four repetitive units (Rep), known as 4RepCT.[87] Andersson et al. developed a novel miniature spidroin that includes the N-terminal domain (NT), 2 repeat units

and the C-terminal domain (NT2RepCT).[88] NT2RepCT is far superior to 4RepCT in production yield and protein solubility and enables biomimetic spinning,[88] as well as the generation of 3D structures. NT2RepCT has not been used in cell culture or *in vivo*, while 4RepCT has been successfully used in both circumstances.[89-93]

Native spider silk and other recombinant forms of spider silk have also been reported to support cell culture and *in vivo* implantation in animal studies.[94-97] Generally, spider silk has been considered biocompatible and immune privileged.[98] Macrophages have nonetheless been found surrounding implanted spider silk in several studies,[91,95] and there have been indications of a more active immune reaction to native spider silk.[94,99] However, the immune response to spider silk, including lymphocytic reactivity, has not been characterized thoroughly, leaving the question of spider silk's immunogenicity unresolved.



Rising et al., Nature Chemical Biology, 2015

Figure 4. Illustration of the basic composition of the spider silk protein.

1.3 IMMUNE TOLERANCE

The history of organ transplantation between humans begins in 1954 with a kidney transplantation between identical twins.[100] The first allogeneic human heart transplantation took place in 1967 and the patient survived 18 days, with the death attributed to acute rejection.[101] Survival post-transplantation was initially very poor, often measured in months, due to immunological rejection.[101,102] Immune tolerance was identified as the key to transplant success and experiments with total-body irradiation were performed, resulting in acceptance of the graft but also in the death of the patient due to bone-marrow aplasia.[102] In the 60's and 70's, cortisone and azathioprine were used for immune suppression and in 1983, Cyclosporine A was introduced.[103] Cyclosporine A revolutionized transplantation, improving the survival of renal grafts from 45% to 82% at two years.[103] In 1994, FK506, also known as tacrolimus, came into clinical use. Cyclosporine A and tacrolimus use the same mechanism of action, inhibiting T cell proliferation through calcineurin inhibition.[104] Calcineurin is a crucial element in enhancing the transcription of interleukin 2 (IL-2) in T cells, which is essential for T cell survival and proliferation after activation.[105] Tacrolimus remains the basis of most immunosuppressive regimes and has beneficial effects on graft survival at the expense of severe side effects, however, including cancer, kidney failure, infections and cardiovascular disease.[106] This has consequences for the usability of cell therapy for chronic diseases, like

diabetes mellitus type 1 or heart failure, since the side effects of immune suppressive therapy will outweigh the advantages of a possible cure for most patients. A selective immune tolerance that leaves the immune response intact to respond to cancer cells and pathogens, while accepting transplanted cells, would be the optimal solution. This selective immune tolerance is the basic concept behind the studies where we induced immunological tolerance by using short-term costimulation blockade. Before we discuss this concept, we need to address the different components of the immune response.

1.3.1 Regulatory T cells

The human immune system is divided into the innate and adaptive components. The adaptive immune cells, T cells and B cells recognize specific antigens through certain amino acid sequences that may bind to their receptor. The receptor is highly variable and the binding thereof induces the proliferation and production of cytokines, with the aim to kill presumed invaders. The innate immune system, including macrophages, complement and NK cells, may also recognize specific molecules, but they are not as dynamic, and generally respond to the command delivered by T cells in the form of cytokines. Since T cells are central both to the function and control of the immune system, their ability to separate "self" from "non-self" is normally implicated in an adequate or autoimmune response to a stimulus, even though other immune cells, like B cells and macrophages, may actually bring about the event.

Thymic selection is a process where, among other things, the T cells' ability to bind self-antigens is assessed. T cells binding self-antigens are deleted or directed toward a tolerogenic profile.[107] Tolerogenic T cells, Tregs, mediate their effect through both cell–cell interaction and cytokine production.[108] Most Tregs are $CD4^+$ cells that acquire their profile in the thymus.[107,109] However, Tregs may also be generated from naïve $CD4^+$ T cells in response to the ligation of the T cell receptor when other inflammatory stimuli are not present.[110]

Tregs were initially identified in 1995 as $CD4^+$ T cells expressing CD25,[111] a subunit of the IL-2 receptor. A few years later, a mutation of the transcription factor forkhead box 3 (Foxp3) was found to be the reason for the severe autoimmune disorder Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX).[112] Foxp3 has since then been identified as the central element in the development of a tolerogenic profile in T cells.[113] IPEX is the consequence of Treg depletion and resembles the phenotype of severe GvHD or graft rejection, but directed to the patient's own body.[114]

1.3.2 T cell costimulation

As described above, T cells are activated after binding their specific antigen. However, antigen binding is not adequate for T cell activation and a second signal through costimulatory molecules is essential.[115] For costimulation to take place, the DC and T cell must interact closely and the $CD4^+$ T cell must recognize its antigen presented on the Major Histocompatibility Complex (MHC) class II molecule of the DC. After this interaction, there are two main outcomes. If inflammatory stimuli are present, for example, toll-like receptor (TLR) ligation or inflammatory cytokines, the DC expresses costimulatory molecules, most

importantly CD80/86, which bind CD28 on the T cell. Successful costimulation results in a mature effector T cell. In the second scenario costimulatory molecules are absent on the DC, generally due to a non-inflammatory environment, and this may induce anergic T cells or T cell development into a Foxp3^{+} Treg.[116] The need for an inflammatory environment as well as T cell antigen recognition for the generation of effector T cells may protect against inappropriate T cell activation and autoimmunity.

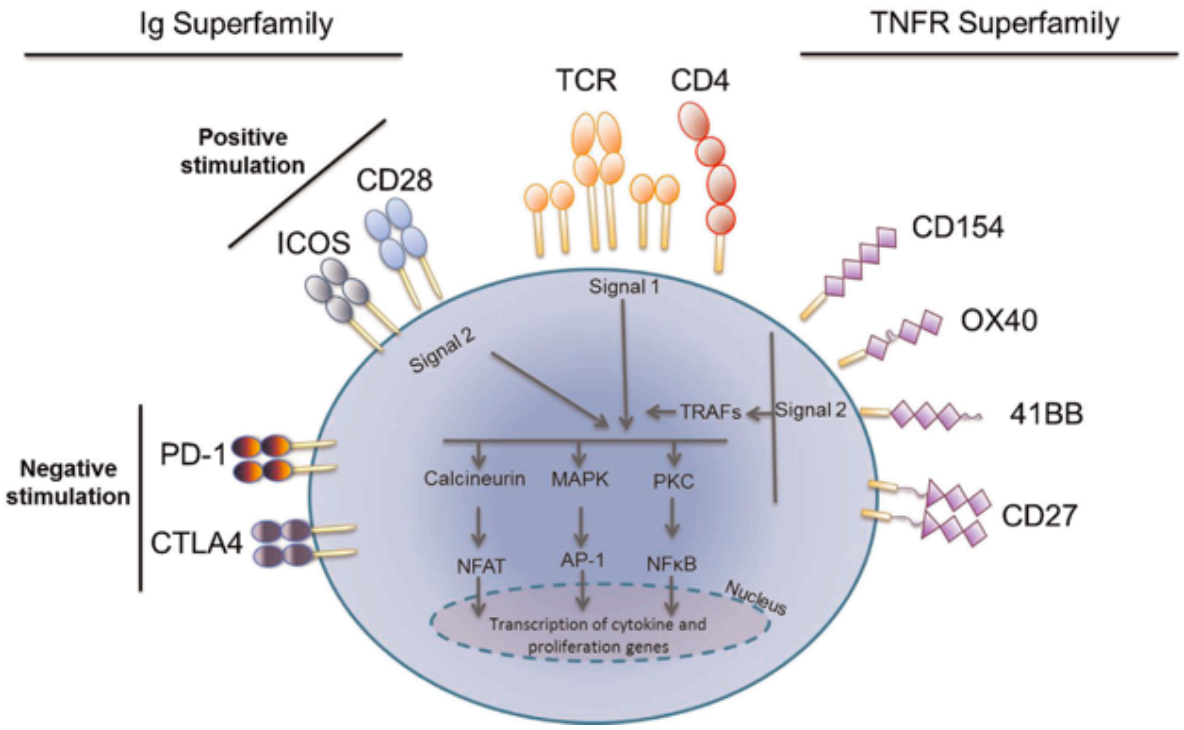


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Figure 5. An overview of important molecules in the costimulation of T cells. Both negative and positive signals are involved, resulting in the initiation or inhibition of overlapping pathways of importance for cell proliferation and cytokine production.

There are several different costimulatory molecules that are important in T cell activation one way or the other, wherein CD80/86-CD28 and CD40-CD40L, along with the formation of a functional immunologic synapse for DC – T cell interaction, can be viewed as central and will be further discussed in the following paragraphs.

1.3.3 CTLA4Ig

The CD28 receptor on the T cell is essential to costimulation. When the T cell receptor is ligated, CD28 will bind CD80/86 on the DC and a strong proliferative signal is initiated within the T cell, resulting in increased IL-2 production.[115] Simultaneously, a negative feed-back loop is activated where cytotoxic T-lymphocyte-associated protein 4 (CTLA4) is brought to the surface of the T cell. CTLA4 also binds CD80/86, but with higher affinity than CD28[117] and inhibits prolonged proliferation by removing CD80/86 from the DC surface through endocytosis, thereby blocking further costimulation.[118] CTLA4 is also expressed by Tregs and is essential for tolerance induction[119] through the removal of costimulatory molecules and

possibly also through the induction of IDO expression and other tolerogenic pathways in DCs.[108]

In searching for alternatives to using calcineurin inhibitors as immunosuppressive treatment agents, a fusion protein consisting of the extracellular domain of CTLA4 and the Fc region of a human Immunoglobulin G1 (IgG1) molecule, CTLA4Ig, was created. The aim was to block T cell costimulation and thereby avoid the rejection of transplanted organs by inducing T cells that were anergic and/or tolerogenic toward the alloantigens derived from the donor. The first version, abatacept, was further refined into belatacept, with enhanced affinity for CD80/86.[120] Belatacept has been used as a substitute for tacrolimus in kidney transplantation with reduced nephrotoxicity and a better cardiovascular profile at the cost, however, of an increased risk of acute rejection.[121] Belatacept has also been evaluated for liver transplantation, giving rise to an increased risk of graft loss and mortality and resulting in the termination of the clinical trial.[122] Today, belatacept is mainly considered for kidney transplant patients who are afflicted by the side effects related to tacrolimus and there are few clinical trials with belatacept concerning the transplantation of other organs.[123] The increased acute rejection has been attributed to $CD28^-$ T cells, independent of costimulation through the interaction of CD28 and CD80/86. $CD28^-$ T cells are not present at birth, but are found to increase with age in humans.[124] $CD8^+CD28^-$ T cells are frequently occurring and include regulatory T cells,[125] whereas the $CD4^+CD28^-$ T cell population is less common and accumulates in inflammatory disorders.[126] The latter seem to make up a senescent T cell population, which is often involved in autoimmunity.[127] These $CD4^+CD28^-$ T cells are likely to increase the risk of belatacept-resistant rejection, as it has been shown that accumulation of a subset of these cells will increase the risk of kidney transplant rejection in patients on belatacept but not tacrolimus treatment.[128]

1.3.4 Anti-CD40L

While the costimulatory molecule CD28 is constitutively expressed by naïve T cells, CD40L is induced when the T cell binds its ligand. Mature DCs, exposed to inflammatory stimuli, express CD40, which binds CD40L, resulting in both enhanced T cell proliferation and the stimulation of the DC to produce proinflammatory cytokines.[129] The antibody-mediated blockade of CD40L was initially promising with successful animal experiments, including an allogeneic kidney transplant model in non-human primates.[130] where anti-CD40L alone was enough to induce acceptance. In theory, anti-CD40L may work through a dual mechanism with both direct blockage of T cell activation and selective depletion of activated T cells by antibody labeling, which may account for the efficiency observed. CD40L is also present in a soluble form and may be secreted by thrombocytes, where it is involved in the formation of a stable thrombus.[131] Thrombotic events using anti-CD40L were initially reported in non-human primates[132] and subsequently, in humans when a clinical trial conducted in 2003 with systemic lupus erythematosus (SLE) patients was interrupted for this reason.[133] The likely mechanism was anti-CD40L-mediated thrombi destabilization, causing embolus, and the binding of anti-CD40L to activated thrombocytes.[131] Activated thrombocytes display CD40L on their surface and the Fc part of anti-CD40L may bind other thrombocytes, causing aggregation. Since the

recognition of the thrombotic side effects of anti-CD40L, CD40 antibodies have been developed and tested in animal models of transplantation with mixed results.[134,135] Some of the effects observed with anti-CD40L could have been lost due to the loss of the antibody-mediated destruction of activated T cells.

1.3.5 Anti-LFA-1

The integrin lymphocyte function-associated antigen 1 (LFA-1) is important in the adhesion of immune cells, for example, in their migration from the blood stream to target tissues. LFA-1 is also implicated in T cell costimulation, where it binds intercellular adhesion molecule 1 (ICAM-1) to establish the immunologic synapse.[136] This is essential for the tight interaction needed between the DC and T cell for T cell activation.[137] Antibodies directed towards LFA-1 have been effective in the treatment of autoimmune disease and in 2003, efalizumab was approved for the treatment of psoriasis. In 2009, it was withdrawn when several patients developed progressive multifocal leukoencephalopathy (PML) a disease that affects profoundly immune compromised individuals due to the reactivation of the John Cunningham (JC) virus.[138]

PML is also associated with treatment with monoclonal antibodies directed toward the very late antigen 4 (VLA-4), natalizumab, which is used for the treatment of multiple sclerosis.[139] VLA-4 is also an integrin that is important in lymphocyte adhesion, demonstrating the significance of adhesion in immune cell function and also the potency of these drugs.

1.3.6 Costimulation blockade

As described above, the blockade of costimulatory molecules has been investigated for the treatment of autoimmune disease and transplantation with mixed results. It is generally effective, but as with other immune suppressants, the long-term side effects may be severe. The initial hope was that costimulation blockade would be able to mimic the natural tolerance to self-antigens and extend this tolerance to transplanted cells through the selective induction of Tregs. In theory, the total blockade of costimulation at the time of transplantation may induce Tregs specific to transplant-derived peptides. The blockade should be administered transiently and the Tregs specific to the transplant would confer indefinite and specific tolerance, without further immune suppression. This would constitute an entirely new way to address rejection, wherein long-term immune suppression could be avoided.

For allogeneic transplantation in mice, transient costimulation blockade combining CTLA4Ig and anti-CD40L have been effective in some settings,[140] but more stringent immunological models have revealed that graft survival is not indefinite.[141] The addition of anti-LFA-1 has been shown to facilitate xenograft survival in mice, including HESCs, when combined with CTLA4Ig and anti-CD40L.[142-144] However, when translated to non-human primates, long-term allograft survival (years) with short-term costimulation blockade has not been achieved.[145-147] The more complex immune system of primates and their longer life-span, along with exposure to different antigens and infections, may explain these observations. Infections increase the risk of heteroimmunity, meaning the pre-existence of memory cells specific to the transplant, due

to similar antigens on the pathogen and transplant. Further studies are needed to better understand the mechanisms behind the prolonged graft survival achieved with transient costimulation blockade in animal models.

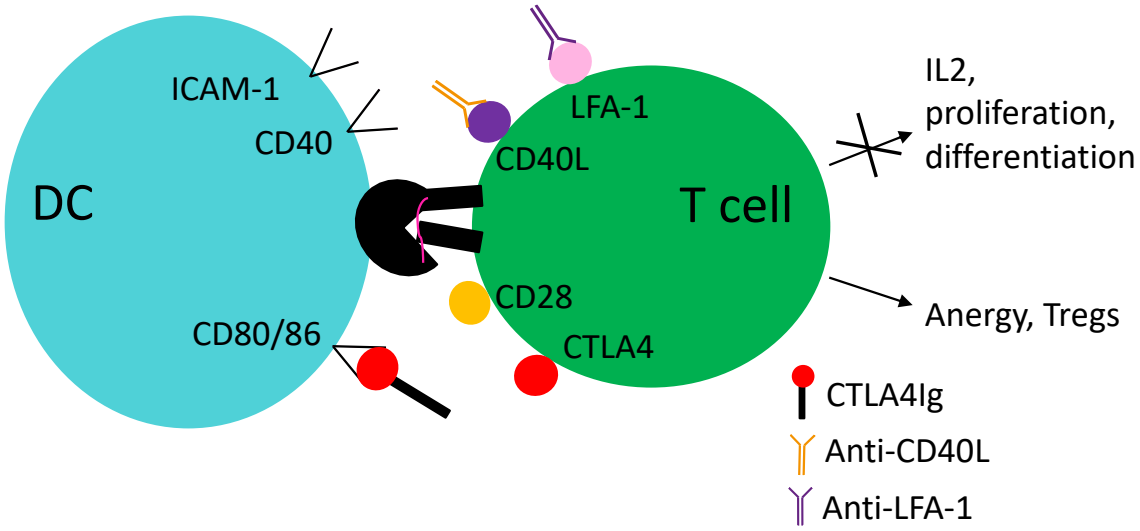

Figure 6. Schematic overview of how a costimulation blockade may interrupt the interaction between a T cell and a DC.

In this doctoral thesis I have considered different ways to enhance cell retention in the tissue. Costimulation blockade, CTLA4Ig and anti-CD40L with or without anti-LFA-1 were used to avoid rejection without continuous immune suppression. Matrigel or a new kind of artificial spider silk, NT2RepCT, supplied anchorage for the cells to avoid anoikis. In the first two papers, the immune reaction was studied both histologically and functionally and in the last two papers, strategies to avoid anoikis were combined with costimulation blockade to enhance engraftment.

2 AIMS

The overall aim of this thesis was to identify and explore methods to improve cell engraftment in xenogeneic and allogeneic cell transplantation. The specific aims of the studies included are stated below.

STUDY

I. To further explore the possibilities and mechanisms for the acceptance of human embryonic stem cells in mice with the use of CTLA4Ig, anti-CD40L and anti-LFA-1.
II. To investigate the synergistic effects of MSCs and costimulation blockade on graft acceptance in an allogeneic animal model.
III. To investigate *in vivo* retention and the vasculogenic potential of xenogeneic human fetal cardiac MSCs in the subcutaneous tissue of mice after a transient course of CTLA4Ig, anti-CD40L and anti-LFA-1 treatment.
IV. To explore the biocompatibility of NT2RepCT when it is used as a 3D scaffold for human fetal cardiac MSCs *in vitro* and *in vivo*.

3 MATERIALS AND METHODS

3.1 ETHICAL APPROVAL

Human fetal tissue was obtained after the donor's informed consent and approval by the Regional Ethics Board in Stockholm, 2013/457-31/4 and 2015/1369-31/2 (paper III and IV).

All animal experiments were approved by the Linköping Ethical Committee on Animal Experiments with ethical approval numbers S198-11 and S31-12 (Paper I), S 76-11 and S16-13 (Paper II) and S31-14 and S22-15 (Paper III and IV).

3.2 2D CELL CULTURE AND CELL EXPANSION (PAPER III AND IV)

The hfcMSCs were isolated through the collagenase digestion of fetal heart tissue in week 6 to 10. The cells were seeded onto plastic culture dishes and the adherent cells, the mesenchymal fraction, were kept and expanded on Geltrex-coated culture plates in DMEM/F12 supplied with 2% fetal bovine serum (FBS) and B27 as well as 100ng/ml Wnt3a and 10ng/ml Epidermal Growth Factor (EGF). At passages 6 to 10, the cells were analyzed or used in experiments. The hfcMSCs were characterized earlier and fulfill the criteria for MSCs established by the International Society for Cellular Therapy (ISCT).[59]

After expansion, the cells were re-seeded in culture dishes either straight onto the cell-culture-treated plastic surface or onto dishes coated with Geltrex or NT2repCT. The cells were given DMEM/F12 supplied with 2% FBS and B27. After five days, the cells were removed from the culture dish and counted using Trypan blue 0.4% and automated cell counter Countess® (Thermo Fisher Scientific). Cell death was assessed in the culture dish with Propidium Iodine (PI) staining and the number of dead cells per well was estimated using a fluorescence microscope.

3.3 MATRIGEL 3D CULTURES (PAPER III)

The hfcMSCs in DMEM/F12 were mixed with Matrigel Matrix at a 1:1 ratio to produce a suspension with 2,000 cells/µl. Fifty µl was seeded as a hanging drop and allowed to polymerize. After 10 days, the 3D cultures were used for further analysis.

3.4 ARTIFICIAL SPIDER SILK SCAFFOLDS WITH 3D CULTURES (PAPER IV)

Thin NT2repCT fibers were modeled into 3D structures, referred to as fiber nests. The fiber nests were divided into pieces, or scaffolds, with a diameter of approximately 5 mm to make implantation feasible. The scaffolds were placed at the bottom of a non-adhesive test tube,

which restricted the attachment of the cells to the NT2RepCT scaffold. The hfcMSCs were seeded on top of the scaffold and DMEM/F12 supplied with 2% FBS and B27 or endothelial differentiation medium (Lonza) was added. The 3D cultures were harvested at 2 weeks for *in vivo* implantation. The 3D cultures dedicated to *in vitro* analyses were snap-frozen in Optimal Cutting Temperature compound (OCT) at 2 to 8 weeks.

3.5 ANIMAL EXPERIMENTS

All mice were purchased from Scanbur or Taconic Biosciences.

For Paper I Nod Scid Gamma (NSG), Scid/Beige, C57bl/6 and Balb/C mice were used. The mice were anaesthetized with continuous isoflurane and (3-5) x 10^6 HESCs re-suspended in PBS were injected under the kidney capsule.

For Paper II C57bl/6 and Balb/C mice were used. The C57bl/6 mice were rendered diabetic by 75 mg/kg Alloxan treatment. Allogeneic insulin producing islets were delivered through the portal vein alone or together with autologous MSCs. The mice were given either CTLA4Ig alone, CTLA4Ig with anti-CD40L or isotype control antibodies (human IgG and hamster IgG) as an intraperitoneal injection on the day of transplantation (d0) and every other day until postoperative day 10.

For Paper III, NMRI mice were implanted with hfcMSCs as a subcutaneous injection behind the ear. The cells were re-suspended in Matrigel or DMEM/F12. Continuous isoflurane was used to anaesthetize the mice during the procedure.

For Paper IV, NMRI mice were implanted with NT2repCT 3D scaffolds with or without hfcMSCs cultures attached. The mice were anaesthetized with continuous isoflurane and an incision behind the ear was made where the 3D culture could be inserted into the subcutaneous fat. One or two stitches were used to close the wound.

All immunocompetent mice that were implanted with human cells (Paper I, III, IV) received a combination of CTLA4Ig, anti-CD40L and anti-LFA-1 or isotype control antibodies (human Fc-IgG1, hamster IgG and rat IgG2a) as an intraperitoneal injection on the day of transplantation and every other day until postoperative day 6.

All mice were euthanized through neck dislocation and kidney (Paper I), liver (Paper II) or subcutaneous grafts (Paper III and IV) that were excised, embedded in OCT and snap-frozen. When a mixed lymphocyte reaction was performed the spleen was also harvested.

3.6 MIXED LYMPHOCYTE REACTION (PAPER II AND III)

The mixed lymphocyte reaction (MLR) was performed to further evaluate whether costimulation blockade induced immunologic tolerance. The same method was also used to

validate the quality of the costimulation blockade antibodies when a new batch was obtained (Paper III). Irradiated splenocytes from naïve Balb/C mice were used as stimulators. The C57bl/6 naïve splenocytes or splenocytes from animals in different treatment groups (Paper II) were used as responders. Stimulator and responder splenocytes were co-cultured for 3 days and subsequently pulsed with [H^3] thymidine. After 18 h, radioactive incorporation was counted to estimate the number of cell divisions. When MLR was applied to test the costimulation blockade antibodies, the antibodies were added to the coculture to verify their inhibitory effect.

3.7 FLUORESCENT IN SITU HYBRIDIZATION (FISH) (PAPER I, III AND IV)

The cryo-sections were stained with hematoxylin and eosin (H&E) to localize the area of injection or the NT2repCT scaffold. Sections close to or within possible grafts were fixed in 4% formaldehyde and antigen retrieval was performed by boiling the tissue sections in citric buffer. The slides were subsequently digested with pepsin (100μg/ml) in 0.01M HCl at 37°C and the hybridization probe (07J04-005, Abbot) was added, after which the slides were heated to 74°C to achieve hybridization. The slides were incubated over night at 37°C and then washed in saline-sodium citrate (SSC) buffer with 0.3% Igepal at 72°C before they were dried and stained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) or subsequently used for immunohistochemistry.

3.8 IMMUNOHISTOCHEMISTRY

Snap-frozen tissue or 3D cultures in OCT were sectioned into 5 μm sections. For Aggrecan staining, paraffin embedded cell-clusters were used. The tissue sections were fixed in either 4% formaldehyde in PBS, cold ethanol or cold acetone. Antigen retrieval was performed when necessary either by treating the slides with 0.3% Triton in PBS or Heat Induced Antigen Retrieval (HIER), which we achieved by boiling the slides in citric buffer. Primary antibodies against CD4 (YTS191.1), CD8 (KT15), Foxp3 (FJK-16S), CD31 (JC70A and polyclonal rabbit), αSMA (1A4 or polyclonal rabbit), Troponin T (1C11), Vinculin (hVIN-1), Collagen IV (polyclonal rabbit), Perlecan (A71), Laminin γ1 (2E8), Laminin α4 (CL3183 and polyclonal rabbit), Laminin α5 (polyclonal rabbit), Fibronectin (polyclonal rabbit) and Aggrecan (AF-28) were used. Secondary antibodies goat anti-Rabbit IgG (AF488), rabbit anti-Mouse IgG (AF488), donkey anti-Rat IgG (AF488) or donkey anti-Rabbit IgG (AF546) were chosen depending on the origin of the primary antibody.

Immunohistochemical staining was also performed on cells and Matrigel 3D cultures fixed in the culture dish. The presence of CD31, αSMA or Troponin T was assessed using the antibodies listed above.

3.9 TAQMAN PCR (PAPER III)

The hfcMSCs were harvested after expansion to assess mRNA expression. Commercially available Human Aortic Endothelial Cells (HAEC) and Human Carotid Artery Smooth Muscle Cells (HCtASMC) were analyzed together with hfcMSCs as a control and for comparison.

A Pico Pure RNA Isolation kit was used to extract RNA from the cells and a High Capacity cDNA Reverse Transcription kit was used to produce cDNA. Gene assays for CD31 (Platelet Endothelial Cell Adhesion Molecule – PECAM1), αSMA (ACTA2) Troponin T (TNNT2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used in the PCR reactions. To calculate the ratio between the gene of interest and the endogenous control (GAPDH), we used the comparative ΔCt method.

3.10 STATISTICAL ANALYSIS

For Paper II, one-way analysis of variance followed by the Tukey-Kramer post hoc test was applied to analyze the data. The Kaplan-Meier method was used to visualize animal survival and the log-rank test was used for comparisons between the treatment groups.

For Paper III and IV, the Kruskall Wallis test with Dunn's correction for multiple comparisons was used when analyzing ≥ 3 groups and the Mann-Whitney U-test was used for 2 groups.

The value of $p<0.05$ was considered to indicate a significant difference.

4 SUMMARY OF RESULTS

4.1 PAPER I AND II – IMMUNOLOGY

4.1.1 Costimulation blockade allows HESC engraftment in mice (Paper I)

Immunocompetent mice, Balb/C and C57bl/6, along with immunodeficient mice, Scid/beige and NSG, were transplanted with HESC line HS181 under the kidney capsule (Table 1). After 6 to 8 weeks the presence of human cells was assessed using FISH that identified human DNA. NSG mice are severely immunodeficient and lack both adaptive and innate immune functions, including functional NK cells, and are known to accept human cells[148]. These mice were used as a positive control, where two out of two mice accepted HS181 and displayed large teratoma-like grafts, establishing the model as feasible. The immunocompetent mice and Scid/beige mice were subsequently transplanted with HS181 and treated with CTLA4Ig, anti-CD40L and anti-LFA-1 (costimulation blockade) or isotype control antibodies every other day during the first week after transplantation. Scid/beige mice were included due to their immunodeficiency to ensure that the antibodies did not interfere with engraftment and to ascertain cell viability. Scid/beige mice lack adaptive immunity but display functional NK cells.[149] To our surprise, none of the Scid/beige mice accepted HESCs; instead 3 out of 12 immunocompetent mice treated with costimulation blockade did. The engrafted HESCs formed teratoma-like structures, similar to what was seen in the NSG mice. The immunocompetent mice treated with isotype control antibodies served as negative controls and no surviving HESCs were identified in these animals, which agrees with the findings of earlier studies.[144]

	Positive	*Negative*	*Total*
HS181 in NSG	2	0	2
HS181 in Scid/beige+costimulation blockade	0	3	3
HS181 in Scid/beige+control antibodies	0	3	3
HS181 in Balb/c, C57bl/6+ costimulation blockade	3	9	12
HS181 in Balb/c, C57bl/6+ control antibodies	0	12	12
HS360 in C57bl/6+costimulation blockade	2	0	2
HS360 in C57bl/6+control antibodies	0	2	2

NSG, NOD Scid gamma.

Table 1. Human embryonic stem cells transplanted into different mouse strains.

In order to further support our findings a different HESC line, HS360, was transplanted to immunocompetent mice. Two out of two mice treated with costimulation blockade displayed surviving human cells. The mice treated with isotype control antibodies rejected HS360.

4.1.2 Costimulation blockade and MSCs synergize for survival of allogeneic islets in mice (Paper II)

To study whether allograft survival in mice may be improved by combining CTLA4Ig and anti-CD40L with MSCs, a diabetic mouse model was used. The C57bl/6 mice were rendered diabetic and given insulin-producing islets of Balb/C origin. The islets were injected to the liver via the portal vein with or without autologous MSCs. The mice were treated with CTLA4Ig, CTLA4Ig + anti-CD40L or isotype control antibodies (Table 2). The blood glucose levels were measured daily, and two 2 consecutive days showing a glucose level >20 mM was considered a rejection.

MSCs combined with CTLA4Ig and anti-CD40L significantly increased graft survival, in comparison to all other groups, to > 100 days for every recipient (Fig. 7A). Thirty days after transplantation, a glucose tolerance test was performed to test graft function. This demonstrated that mice treated with MSCs, CTLA4Ig and anti-CD40L had blood glucose levels equivalent to non-diabetic mice at 90 min post-exposure, with glucose levels significantly lower than those of mice treated with only costimulation blockade (Fig. 7B). Insulin mRNA in the liver of recipients treated with MSCs, CTLA4Ig and anti-CD40L was found to decrease at 100 days post-transplantation compared to at 30 days, but was still significantly higher than for the other treatment groups.

Group	MSC	Treatment[a]	Mice (n)	Graft survival (days)	MST (mean ± SD)
A	Negative	Control	6	3, 6, 8, 8, 8, 9	7 ± 2.19
B	Negative	CTLA4Ig	6	8, 13, 17, 37, >100 ×2	45.8 ± 43.1
C	Negative	CTLA4Ig + anti-CD40L	9	4, 7, 34, 48, >100 ×5	65.89 ± 42.5
D	Positive	Control	6	7, 8, 12, 8, 8, 10	8.83 ± 1.83
E	Positive	CTLA4Ig	5	12, >100 ×4	82.4 ± 39.4
F	Positive	CTLA4Ig + anti-CD40L	6	>100 ×6	>100

[a]CTLA4Ig + anti-CD40L: 0.5 mg per mouse i.p. (day 0); 0.25 mg per mouse i.p. (days 2, 4, 6, 8, 10).
Abbreviations: MSC, mesenchymal stromal cell; MST, mean islet graft survival time.

Table 2. Experimental groups and islet graft survival.

4.1.3 Foxp3$^+$ T cells surround surviving allogeneic and xenogeneic cells in immunologically tolerant mice (Paper I and II)

To understand the nature of immunologic acceptance of the allogeneic and xenogeneic grafts described above, tissue sections from recipients with surviving grafts were stained for CD4, CD8 and Foxp3. In study II, allogeneic islets were engrafted in the mice treated with MSCs, CTLA4Ig and anti-CD40L and in study I, the xenografts of HESCs survived under the kidney capsule in costimulation-blockade-treated animals. A similar finding in both studies, was that

the allo- and xenografts were surrounded by Foxp3$^+$ cells at the border between the graft and the host. Treatment with only CTLA4Ig and anti-CD40L resulted in allografts being more scarce in Foxp3$^+$ cells and CD4/CD8 infiltration dominated.

4.1.4 TGFβ mRNA is increased in the liver of mice with engrafted allogeneic islets at 100 days (Paper II)

TGFβ, IDO and Foxp3 mRNA levels were assessed in the liver of allogeneic islet recipients to further analyze the immunologic response to the surviving grafts. TGFβ is a cytokine with immunosuppressive properties and is associated with Treg transformation. TGFβ mRNA was upregulated in all animals with surviving islets at 100 days post-transplantation when compared to 30 days post-transplantation.

IDO is an immunomodulatory molecule produced by MSCs[37] and tolerogenic DCs[42] as a response to inflammation. IDO mRNA was measured 100 days after transplantation and found in recipients treated with MSCs, CTLA4Ig and anti-CD40L or CTLA4Ig and anti-CD40L with no significant difference. The other groups displayed very low IDO mRNA levels. Considering the difference in infiltrating Foxp3$^+$ cells observed in the tissue sections, mRNA expression in the liver of recipients with functional grafts was assessed at 100 days. No significant difference between mice treated with MSCs, CTLA4Ig and anti-CD40L, compared to only CTLA4Ig and anti-CD40L, could be established.

4.1.5 The addition of MSCs to costimulation blockade attenuates lymphocyte proliferation (Paper II)

MLR was performed 30 and 100 days after the transplantation of allogeneic Balb/C islets to C57bl/6 mice. Irradiated splenocytes from the donor mice were co-cultured with splenocytes from the recipient mice of the different treatment groups. Proliferation was measured using [^{3}H] thymidine incorporation. One-hundred days post-transplantation, the proliferation induced by donor antigen in mice treated with MSCs, CTLA4Ig and anti-CD40L was similar to that of naïve mice and significantly lower compared to mice given CTLA4Ig and anti-CD40L (Fig. 8).

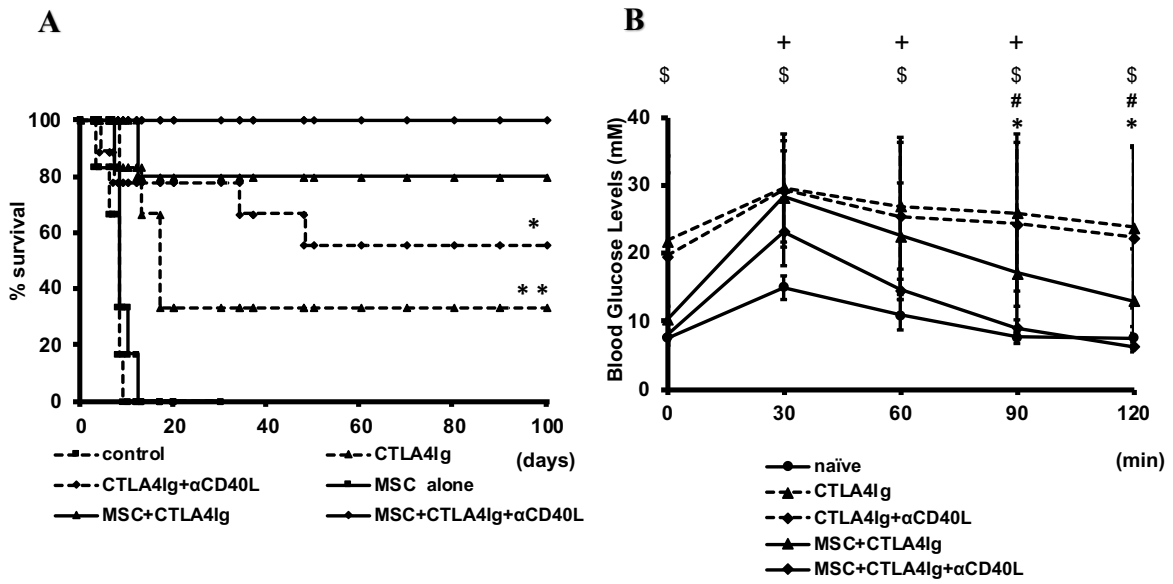

Figure 7. (A) shows the percentage of graft survival in mice transplanted with allogeneic islets. The differences between groups were tested using log-rank statistics. *, $p < 0.05$: MSC+ CTLA4Ig + anti-CD40L vs. CTLA4Ig + anti-CD40L; **, $p < 0.05$: MSC + CTLA4Ig + anti-CD40L vs. CTLA4Ig. (B) depicts the results from the intraperitoneal glucose tolerance test 4 weeks after transplantation. Non-transplanted age-matched C57BL/6 naïve mice were tested simultaneously. Data are shown as mean ± SD. *, $p < 0.05$: MSC + CTLA4Ig + anti-CD40L vs. CTLA4Ig + anti-CD40L; #, $p < 0.05$: MSC + CTLA4Ig + anti-CD40L vs. CTLA4Ig; $, $p < 0.05$: naïve vs. CTLA4Ig; +, $p < 0.05$: naïve vs. CTLA4Ig + anti-CD40L. Statistical analysis was performed by analysis of variance with the Tukey-Kramer post hoc test, n = 5–9.

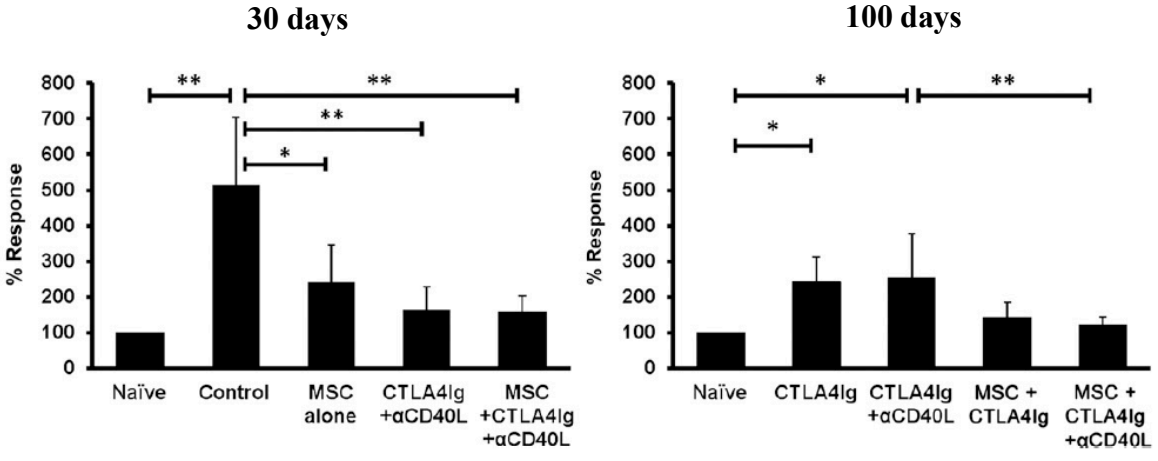

Figure 8. The suppression of T cells' proliferative response at 30 days and 100 days in allogeneic islet recipients. The suppressive effect was assessed using a mixed lymphocyte reaction wherein splenocytes from C57BL/6 recipients were co-cultured with Balb/C splenocytes at 4 days and the proliferation was measured by [^{3}H] thymidine incorporation. The results are shown as a percentage of the response calculated from the mean cpm of the allogeneic reaction after the subtraction of the mean syngeneic response in each group divided by that of naïve mice in each experiment *, $p < 0.05$; **, $p < 0.01$.

4.2 PAPER III AND IV – ENGRAFTMENT

4.2.1 Human fetal cardiac MSCs cultured in 2D express αSMA and low levels of CD31 (Paper III)

Human fetal cardiac MSCs (hfcMSCs) are MSCs originating from the mesenchymal fraction of the fetal heart. These cells can be efficiently expanded in a culture and fulfill the ISCT criteria of being MSCs.[59] The hfcMSCs are multipotent and can be differentiated into endothelial cells, smooth muscle cells and, to a minor extent, spontaneously beating cardiomyocytes.[59] Considering their origin and plasticity, hfcMSCs have the potential to serve as optimal cells for cellular therapy aiming to restore heart function.

In the preparation for the *in vivo* experiments, the hfcMSCs were expanded in 2D cultures. The αSMA expression was found to be high, with an eight-fold increase in mRNA when compared to Human Carotid Artery Smooth Muscle Cells (HCtASMCs), and CD31 expression was limited, with mRNA levels 500 times lower than in Human Aortic Endothelial Cells (HAEC). Troponin T was barely detectable at the mRNA level and immunohistochemistry yielded no positive cells. The immune stainings for αSMA showed ubiquitous expression, whereas $CD31^+$ cells were few in numbers, agreeing with the mRNA findings.

4.2.2 hfcMSCs survive and proliferate on NT2RepCT (Paper IV)

Since we wanted to explore NT2RepCT as a scaffold for *in vivo* implantation we needed to assess whether NT2RepCT is able to support cell culture. The hfcMSCs were cultured in 2D on dishes coated with NT2RepCT, Geltrex (a Matrigel-like coating normally used for hfcMSCs cultures) or no coating (plastic). No difference was found in the cell survival or proliferation of hfcMSCs depending on the coating of the culture dishes, establishing NT2RepCT as suitable to support hfcMSC culture.

4.2.3 hfcMSCs cultured in 3D form tubular structures and express basal membrane components (Paper III and IV)

To simulate the *in vivo* situation, 3D cultures with Matrigel and hfcMSCs were generated. Even though the hfcMSCs formed tubular structures, the expression of CD31, as judged by immunohistochemistry, was low and αSMA dominated. NT2RepCT fibers could be modeled into 3D scaffolds that seemed suitable for implantation. To prepare for the *in vivo* studies we first tested whether NT2RepCT scaffolds may support 3D cultures. The hfcMSCs were seeded on top of an NT2RepCT 3D scaffold and the cultures were analyzed at 2, 4, 6 and 8 weeks. We found that hfcMSCs survived for at least 8 weeks and attached to the NT2RepCT fibers through vinculin. As observed earlier, αSMA was the most prominent staining, but $CD31^+$ cells were also observed. The H&E stainings demonstrated that the hfcMSCs produced their own ECM,

embedding the NT2RepCT fibers. Immunohistochemical stainings indicated that the hfcMSCs produced the basement membrane proteins perlecan, laminin α4 and γ1 as well as collagen IV.

4.2.4 Vessel-like structures are present in 3D cultures of hfcMSCs and NT2RepCT (Paper IV)

Considering the *in vitro* capacity of hfcMSCs to differentiate into endothelial cells[59] and the tubular formation observed in Matrigel 3D cultures, we wanted to investigate the vasculogenic potential of hfcMSCs when cultured on NT2RepCT scaffolds. We found that hfcMSCs seeded onto NT2RepCT scaffolds formed vessel-like structures if supplied with endothelial differentiation medium. Compared to 3D cultures that received basal medium (DMEM/F12 supplied with 2% FBS and B27), the number of $CD31^+$ cells significantly increased (Fig. 9A). We also found that cell density was enhanced (Fig. 9B) and αSMA and CD31 (Fig. 9C) were abundantly expressed along with laminin α4 (Fig. 9D) and fibronectin (Fig. 9E), with the latter two being important components making up the ECM of the vessel wall.[150] The vessel-like structures observed expressed laminin α4 (Fig. 9D) or CD31.

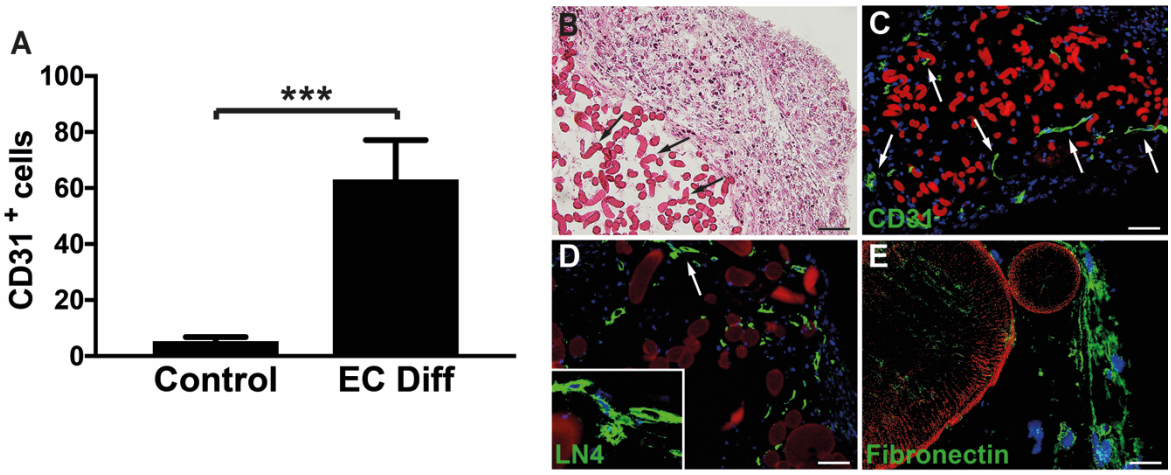

Figure 9. 3D cultures with hfcMSCs on NT2RepCT scaffolds were analyzed with the use of immunohistochemistry after 2 weeks in the culture. The basal medium (control) was compared to a culture in endothelial differentiation medium (EC) regarding the number of $CD31^+$ cells, which is depicted in the bar graph (A). ***P <0.0001. (B–E) are images of tissue sections from 3D cultures grown in endothelial differentiation medium. H&E was used to visualize the ECM formation in relation to the NT2RepCT fibers of the scaffold, which are indicated by the arrows in (B). An immune staining for CD31 (AF488, green) is shown in (C), where positive cells are indicated by arrows. Representative vessel-like structures are shown in (D), here surrounded by Laminin α4 (AF488), where the inset is a magnification of the structure indicated by the arrow. (E) shows an immune staining for fibronectin (AF488). All images (C–E) are merged from microphotographs taken with 546 (red), 488 (green) and DAPI filters. The red appearance of NT2RepCT fibers is due to autofluorescence. The nuclei were stained with DAPI. Scale bars = 60 µm (B, C), 30 µm (D), 10 µm (E).

4.2.5 hfcMSCs are retained subcutaneously in mice treated with costimulation blockade (Paper III and IV)

Immunocompetent NMRI mice were transplanted with hfcMSCs subcutaneously and treated with CTLA4Ig, anti-CD40L and anti-LFA-1 (costimulation blockade) to avoid rejection. The control group received isotype control antibodies. The *in vivo* experiment in study III was performed using Matrigel as the delivery matrix, which was compared to cell delivery in only the culture medium (DMEM/F12). The NMRI mice treated with costimulation blockade and hfcMSCs in Matrigel displayed retaining cells at 4 weeks in 8 out of 8 mice (Fig. 10 A and E), while costimulation blockade and DMEM/F12 yielded retaining cells at 4 weeks in 2 out of 3 mice (Fig. 10 D and H). The number of retaining human cells per tissue section was increased if Matrigel was used for delivery, as compared to DMEM/F12 (Fig. 10 N). The hfcMSCs were not found at 4 weeks when the NMRI mice were treated with isotype control antibodies.

The *in vivo* experiment performed in study IV concerned the transplantation of hfcMSC 3D cultures on NT2RepCT scaffolds. The cells were allowed 12 days to establish 3D cultures on the NT2RepCT scaffolds prior to implantation. At 4 weeks, 3 out of 3 NMRI mice treated with costimulation blockade exhibited surviving human cells (Fig. 12 A and C). The NMRI mice treated with isotype control antibodies displayed occasional human cells in 3 out of 6 animals at 4 weeks (Fig. 12 B and D), but costimulation blockade significantly increased cell survival. Three mice treated with costimulation blockade were kept for 8 weeks, but at that time point no surviving human cells were identified.

4.2.6 The hfcMSCs delivered in Matrigel are rejected after 8 to 12 weeks (Paper III)

Additional groups of NMRI mice, treated with costimulation blockade and implanted with hfcMSCs in Matrigel, were kept for 8 and 12 weeks to assess whether the immunological tolerance of hfcMSCs was persistent. At 8 weeks, 2 out of 6 mice still displayed retaining cells (Fig. 10 B and F). At 12 weeks, human cells were not found in any of the 6 mice kept until this time point (Fig. 10 C, G and M). At 4 weeks, $CD4^+$ or $CD8^+$ cells were not found surrounding or infiltrating the implant (Fig 10 I and L), however at 8 weeks and 12 weeks the surviving cells or Matrigel residues were surrounded by an immune reaction dominated by $CD4^+$ T cells (Fig. 10 J and K). $CD8^+$ and $Foxp3^+$ T cells were present but scarce. The few $Foxp3^+$ cells present were not located close to the graft, as was found in a previous experiment.

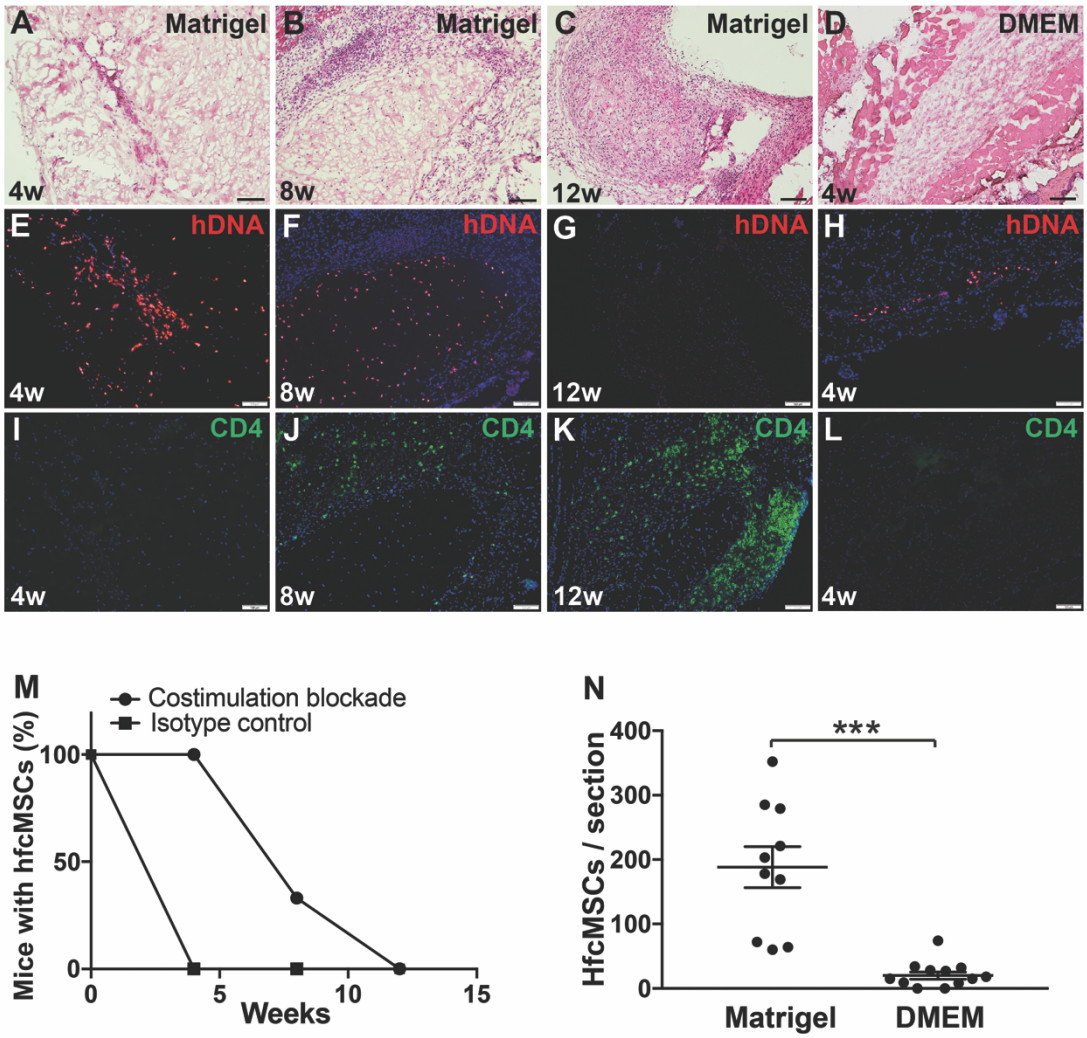

Figure 10. Tissue sections from NMRI mice treated with costimulation blockade (A–L) were stained with H&E (A–D) to visualize the area of implantation, analyzed with FISH (E–H) to assess whether human cells (SpectrumRed) were present and stained with antibodies against CD4 (I–L), AF488 (green), to assess the immunologic rejection. Scale bar = 100 μm. (M) shows the percentage of graft survival over time, in mice implanted with hfcMSCs in Matrigel. (N) is a dot plot visualizing the increased engraftment obtained with Matrigel instead of DMEM/F12 for mice treated with costimulation blockade. ***P < 0.0001. DMEM; DMEM/F12, hDNA; human DNA.

4.2.7 hfcMSCs form vessel-like structures in vivo, expressing CD31 and laminin α4 (Paper III)

As a delivery matrix, Matrigel consistently resulted in hfcMSC grafts at 4 weeks and these grafts were further characterized. H&E indicated the formation of vessel-like structures, which stained positive for laminin α4 or CD31. αSMA was found in areas of close proximity. Immunohistochemistry combined with FISH revealed CD31 and laminin α4 located exclusively close to human cells (Fig. 11 A-D), indicating that these proteins were expressed by hfcMSCs. αSMA was found near both human cells and mouse cells. The hfcMSCs delivered in DMEM/F12 did not express CD31, laminin α4 or αSMA.

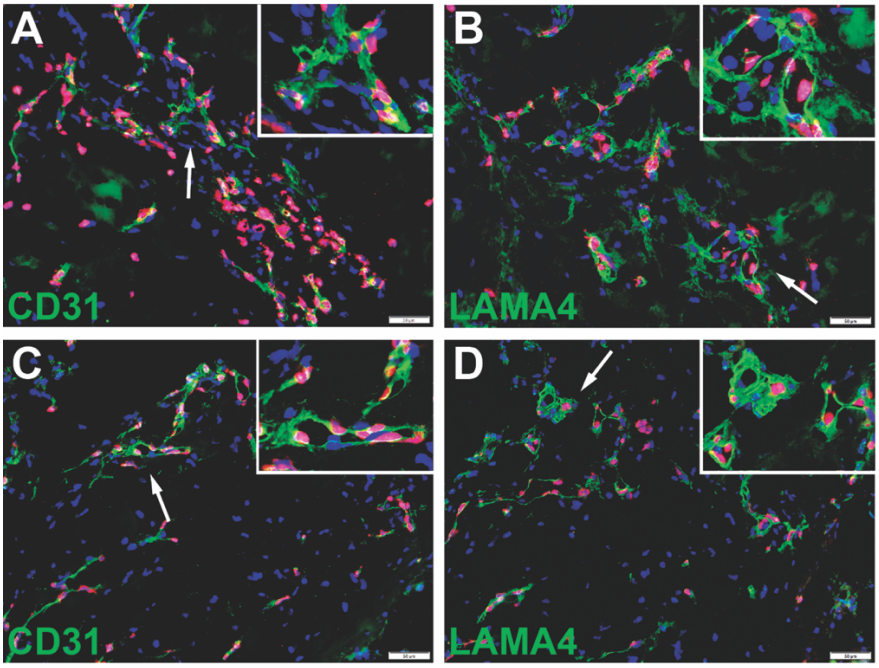

Figure 11. hfcMSCs re-suspended in Matrigel were transplanted to NMRI mice treated with costimulation blockade. FISH was used to assess the presence of cells containing human DNA (SpectrumRed). Immunohistochemistry for CD31 (A, C) and laminin α4 (B, D), both AF488 (green), was applied subsequently to FISH. The arrows indicate the structures magnified in the inset. (A, B) and (C, D) are sections of close proximity. Scale bar: 50 µm. LAMA4; laminin α4.

4.2.8 NT2RepCT 3D scaffolds are immunogenic (Paper IV)

The hfcMSCs on the NT2RepCT scaffolds were surrounded by an immunologic reaction already at 4 weeks post-implantation (Fig. 12 E and F). The $CD4^+$ T cells dominated, but $CD8^+$ T cells were also present as well as occasional $Foxp3^+$ cells. Animals treated with isotype control antibodies displayed a similar immune reaction; however, very few human cells were present. A control group consisting of NMRI mice that received no antibody treatment were implanted with naked NT2RepCT scaffolds. These scaffolds were surrounded by a more intense immune reaction than the one observed in animals implanted with 3D cultures attached to the NT2RepCT scaffolds. Since NT2RepCT is produced in E-coli, we hypothesized that lipopolysaccharides (LPS) originating from the bacteria may be the cause of this observation. In order to solve this problem, different strategies were used wherein NT2RepCT was more extensively washed to remove the LPS, or produced in clear coli, bacteria with defective LPS. At 4 weeks, the NT2RepCT still induced an immunologic response in the subcutaneous tissue of NMRI mice, where both $CD4^+$ and $CD8^+$ T cells were present. The immune response was similar to that observed after the implantation of the original NT2RepCT fibers.

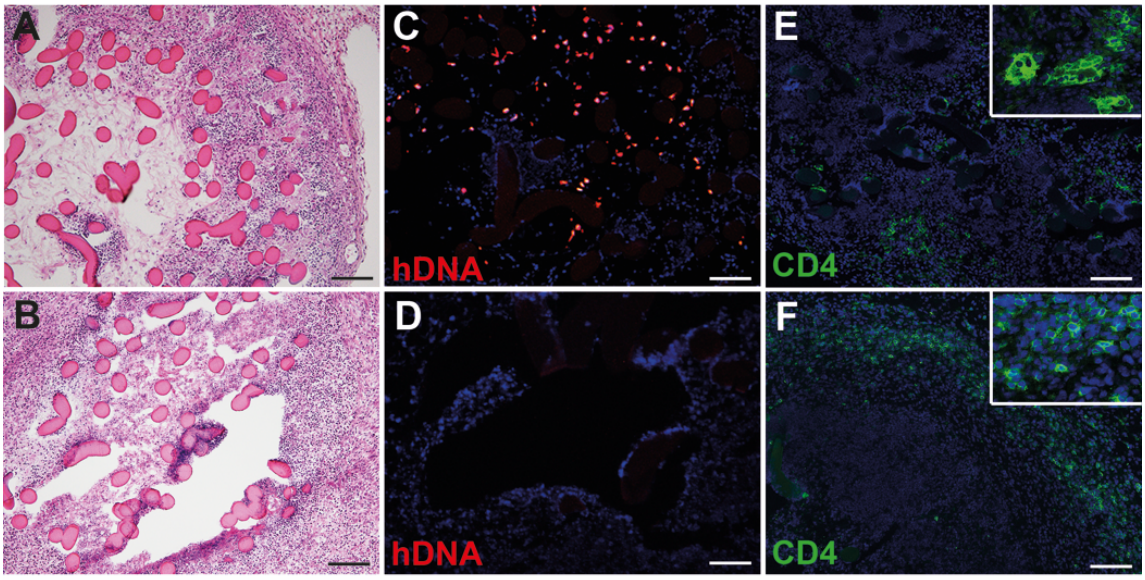

Figure 12. hfcMSC 3D cultures on NT2RepCT were implanted subcutaneously in mice and analyzed after 4 weeks. (A, C, E) show tissue sections from mice treated with costimulation blockade and (B, D, F) show tissue sections from mice treated with isotype control antibodies. H&E (A, B) gave an overview of the tissue and verified the presence of NT2RepCT fibers, staining bright pink. FISH (C, D) identified surviving human cells when they were present (SpectrumRed). Immunohistochemistry for CD4 (E, F), AF488 (green), was applied to assess the presence of lymphocytes. Scale bar = 50 µm (C, D), 100µm (A, B, E, F). hDNA; human DNA.

5 GENERAL DISCUSSION

Immune rejection and anoikis constitute substantial impediments to the successful transplantation of cellular grafts. By addressing these two factors simultaneously, we have demonstrated their synergistic effects on engraftment in different animal models, which was an overall aim of this thesis. Three different cell types have been used for transplantation studies: HESCs, hfcMSCs and insulin-producing pancreatic islets. Previously both MSCs and HESCs have been attributed an immune privileged status, even when used for xenotransplantation, but this notion was later questioned.[151-153] The synthesis of immuno-modulatory molecules by MSCs may potentially contribute to a reduced local inflammation; however, they do not escape immunologic recognition.[153] The absence of MHC class II and costimulatory molecules, a feature often suggested to support immune privilege,[154] only indicate that these cells are not functional APCs. Antigens derived from MSCs and HESCs may still be presented to T cells by host APCs and initiate a specific immune response.

5.1 REGULATORY T CELLS MEDIATE GRAFT ACCEPTANCE AFTER TREATMENT WITH TRANSIENT COSTIMULATION BLOCKADE

A transient blockade of the T cell costimulation has been shown to prevent immune rejection in immunologically competent mice receiving various allografts and xenografts.[142-144,155] Theoretically, the acceptance could be due to the generation of specific Tregs, which is supported by the presence of intragraft T cells in cardiac allografts.[155] In Paper I and II, $Foxp3^+$ Tregs were shown to infiltrate allografts in the liver as well as surrounding the xenografts in the kidney when costimulation blockade was used to avoid immune rejection. These findings further support a central role for Tregs in costimulation-blockade-mediated immunologic acceptance or tolerance. In Paper III, the immunomodulatory effect of costimulation blockade was further challenged by transplanting hfcMSCs to the subcutis of immunocompetent NMRI mice. Grafts were found at 4 weeks, but there were no Tregs in the vicinity. These xenografts were rejected at 8 to 12 weeks. The skin and subcutis are more immunologically active tissues containing numerous APCs, making it harder to achieve persistent acceptance here, as compared to other tissues.[155] The lack of Tregs around the subcutaneous grafts and subsequent rejection show that tolerance was not obtained in this model and further support that immune tolerance induced by costimulation blockade, in contrast to a prolonged acceptance, is mediated through Tregs.

The skin forms a border between the organism and the outer world and is considered a more stringent immunological environment containing more APCs and is also known to be more challenging for transplantation[156] than, for example, the kidney or heart. Furthermore, it might be speculated that pro-inflammatory cytokines produced by the local APCs of the skin reduce

both the formation and stability of Tregs. This could account for the lack of Tregs surrounding the human grafts in the subcutis. Treg instability has been shown for *in vitro*-induced Tregs, which may lose their Foxp3 expression, due to the increased methylation of the Foxp3 gene, compared to thymic Tregs.[157] *In vivo*-generated peripheral Tregs have exhibited a more stable Foxp3 expression with an epigenetic modulation similar to that of thymic Tregs.[41,157] It is possible that Tregs formed in mice treated with costimulation blockade initially display an increased methylation status. Over time the Foxp3 expression could potentially be more stably expressed in the kidney and liver, but perhaps this does not occur in the immunologically more stringent environment of the skin.

It is important to stress that we cannot rule out the rejection of HESCs and islets at a later time point than the one chosen for the studies described in Paper I and II. For the insulin-producing islets transplanted to the liver of allogeneic mice, the decrease in insulin mRNA may indicate the onset of rejection. However, the MLR showed similar T cell reactivity to the allogeneic antigen for transplanted mice as for naïve mice, which instead speaks in favor of long-lasting tolerance.

Another aspect to consider is that the mouse model is not optimal to prove persistent tolerance induction since mice have a short life-span. Even prolonged studies of these animals would be inconclusive, since any therapy applicable to humans would need to induce a tolerance lasting for years or decades.

5.2 HESCS ARE REJECTED BY IMMUNOCOMPROMISED SCID/BEIGE MICE

The HESCs transplanted to immunocompetent mice were subjected to immune rejection, which could be prevented when the costimulation of T cells was blocked. The HESCs transplanted to the kidney capsule of NSG mice formed a teratoma without the need for any immunomodulation. However, the HESCs transplanted to Scid/beige mice did not survive, regardless of costimulation blockade treatment. Scid/beige mice are immunodeficient and lack a functional adaptive immune response; however, in contrast to NSG mice, they do have NK cell function.[148] NK cells may act in xenograft rejection since they recognize MHC class I deficient cells. Foreign MHC class I may not register in a xenogeneic environment, rendering the transplanted cells susceptible to NK cell-mediated destruction. In the case of Scid/beige mice, we cannot expect costimulation blockade to make a difference in terms of cell engraftment, since the mechanism behind costimulation-blockade-mediated immunomodulation depends on a functional adaptive immune response. The lack of Treg formation in Scid/beige mice likely accounts for the observed HESC graft acceptance in immunocompetent mice treated with costimulation blockade, but not in immunodeficient Scid/beige mice. The immunocompetent mice may of course mount a NK cell response as well, but the induction of Tregs, presumably due to costimulation blockade treatment, may dampen the NK cell function. The NSG mice that consistently accepted HESCs lack functional NK

cells,[158] which supports the theory that NK cells are the main reason behind the higher acceptance of HESC in immunocompetent mice with costimulation blockade than in Scid/beige mice.

5.3 IMMUNOLOGICAL LIMITATIONS

Preclinical models utilizing small animals are vital to research and can optimize the use of large animals, such as non-human primates, without increased risk for future patients. However, a mouse model used for immunological studies has several limitations. From an evolutionary perspective, the adaptive immune system developed initially in vertebrates and is therefore a relatively "new" function. It has been the subject of great evolutionary pressure since humans started living in larger communities, with greater potential for the spread of bacterial and viral disease. Perhaps this is part of the explanation for the autoimmune diseases that afflict humans today.

Whereas many other organs are similar in mice and humans, major differences between mouse and human immunology also exist, one example being the accumulation of $CD28^-$ T cells in human adults.[127] Even non-human primates differ substantially from humans, as exemplified by a phase 1 drug trial performed in 2006 with a CD28 superagonist (TGN1412).[159] An orthologous antibody had previously been successfully tested in small-animal models of autoimmune disease[160] and subsequently, TGN1412 had been tested in non-human primates without adverse events.[161] However, when the substance was administered to six healthy human volunteers, they became critically ill due to a cytokine storm and developed organ failure, demanding hospitalization. Evolutionary pressure may explain the great difference between species regarding the immune system and may also indicate large individual variances in the population, further complicating matters.

Further limitations connected to the use of mice include their limited exposure to antigens in general, which is due to a short life span combined with the clean conditions of an animal facility. This limited antigen exposure will reduce the risk of preformed memory immune cells directed to donor-derived antigens, which may facilitate the immune acceptance of transplants. Transplantation is a very special immunologic situation, wherein a large number of cells from the donor is delivered to the recipient. This results in a an extremely high amount of donor-derived antigen becoming available to the immune system. The amount of antigen present shortly after a transplantation would seldom be observed in nature and may override the requirement of costimulation for T cell activation in certain T cell subsets. CD28 stimulation is generally needed for an adaptive immune response since it lowers the threshold for T cell activation after antigen exposure. However, for memory T cells, this threshold is lower to begin with.[162] The overload of antigen derived from the transplant in the period after transplantation could prove to be sufficient on its own for the activation of pre-existing memory T cells, rendering costimulation blockade ineffective. In comparison to humans, the absence of relevant

memory T cells as well as the differential CD28 expression in mice[127] render the discrepancy in results between a mouse model and a human model with costimulation blockade significant. Although mouse models have several drawbacks, they are still extremely valuable in serving as the first *in vivo* model to test a concept derived from *in vitro* experiments.

5.4 SYNERGISTIC EFFECTS OF COSTIMULATION BLOCKADE COMBINED WITH MSCS OR MATRIGEL FOR ENHANCED CELL RETENTION

As the delivery matrix, Matrigel provided increased survival of human cells in a xenogeneic environment, if combined with costimulation blockade treatment. Matrigel alone did not improve cell engraftment and costimulation blockade alone provided significantly smaller grafts. The synergistic effect observed is likely a combination of reduced anoikis during the first days after the delivery due to the presence of Matrigel, and prolonged survival due to immune-modulation mediated by the costimulation-blocking antibodies.

When allogeneic insulin-producing islets were transplanted to the liver in mice, we observed a synergy between costimulation blockade and MSCs. Only mice treated with MSCs in combination with costimulation blockade demonstrated long-term acceptance of allogeneic islets and also retained similar glucose levels to those of healthy mice. As demonstrated by MLR, these mice also showed the same reactivity toward allogeneic antigens as naïve mice. The synergistic effect observed between MSCs and costimulation blockade treatment could potentially be ascribed to a dual effect on the immune response, wherein the MSCs may act through the modulation of innate pathways and the costimulation blockade regulates the adaptive response. Mice that were given islets combined with the administration of MSCs and no additional immunomodulation displayed the same poor islet survival as control animals. However, the findings from the MLR indicated a reduced reactivity toward allogeneic antigens by mice treated with only MSCs as compared to control animals, even though the islets did not engraft. Another potential mechanism is that MSCs decrease anoikis by providing paracrine signals leading to decreased apoptosis, while costimulation blockade decreases immune rejection. An earlier study focused on the trophic effects of MSCs, showing that they can aid in the engraftment of islets in mice.[163] The mechanism proposed depended on the presence of antiapoptotic and proangiogenic factors produced by MSCs. It is reasonable to assume that the positive effects observed when transplanting islets together with MSCs could involve both immunologic and anti-apoptotic mechanisms. The MSCs may reduce anoikis by providing ECM factors that are vital for anchorage and perhaps also cell–cell interactions. The acute inflammatory reaction occurring after delivery into a new tissue could be attenuated earlier by the MSCs' ability to affect the DCs in the vicinity and in this way induce a tolerogenic profile.[36] These results indicate that autologous MSCs may increase the potential of other immune modulatory therapies used in organ or cell transplantation.

5.5 VASCULOGENIC POTENTIAL OF HUMAN FETAL CARDIAC MSCS

The hfcMSCs were studied in a xenogeneic model where long-term survival was not achieved. However, in the case of MSCs as a potential cell therapy for heart disease, long-term survival is not required since the paracrine factors contributing to a positive tissue homeostasis constitute their proposed mode of action. After a myocardial infarction, the original ECM is altered and the new ECM has been shown to contain an increased proportion of Collagen I, while laminins almost disappear and fibronectin content is decreased.[164] This will result in increased tissue stiffness, which may contribute to preventing the rupture of the ventricle wall at the price of lost contractile function and the risk of re-entry ventricular arrhythmias.

In Paper III, we observed that the hfcMSCs formed vessel-like structures expressing CD31 and also that they deposited laminin α4 *in vivo*. In Paper IV, we found that *in vitro* 3D cultures of hfcMSCs on NT2RepCT cultured in an endothelial differentiation medium were able to form $CD31^+$ vessel-like structures, resembling what they did *in vivo*. The cells could also deposit laminin α4 and fibronectin, further supporting their ability to contribute ECM components of relevance after a myocardial infarction. Fibronectin and laminin α4 are important parts of the vascular basement membrane.[150] Therefore, the presence of these proteins along with $CD31^+$ hfcMSCs support the vasculogenic potential of these cells. Since the lack of laminin α4 has been implicated in heart failure in a mouse model,[69] the repeated findings that hfcMSCs deposit this laminin chain both *in vitro* and *in vivo* could be important when considering hfcMSCs as a potential treatment for heart failure. The results presented here indicate that hfcMSCs may have potential for use in cell therapy aiming to treat heart disease. Unpublished results show that Matrigel and costimulation blockade aid in the survival of hfcMSCs in the heart muscle as well, but further studies to assess the functional effects are needed to evaluate whether this kind of treatment may be effective.

A prolonged retention of the transplanted cells for up to a few weeks or months may be sufficient to break a vicious cycle of inflammation and stimulate angiogenesis or other positive regenerative effects in the tissue. For this purpose, we have shown that short-term costimulation blockade was sufficient to keep the implanted hfcMSCs in our subcutaneous animal model. However, the immune rejection observed in the subcutis at 8 to 12 weeks would be deleterious if it took place in the heart muscle. Our unpublished data show that hfcMSCs delivered in Matrigel are present at 4 weeks but not at 8 weeks after transplantation to the heart of NMRI mice treated with costimulation blockade. In the heart, which is not as immunologically stringent as the subcutis, signs of immune rejection with lymphocyte infiltration were not observed at 4 or 8 weeks. Due to the dynamic mechanical environment the cardiac tissue poses a challenge in terms of successful cell anchorage after transplantation and co-transplanted Matrigel is likely not enough to fully counteract anoikis in this model.

5.6 ARTIFICIAL SPIDER SILK SUPPORTS 3D CULTURES

Traditionally, cells have been cultured in 2D and passaged to a new culture plate when they are confluent. This is practical, but the reduced complexity of a 2D model will only reflect cellular processes, like morphogenesis, to a limited degree.[165] The ECM produced will also be disrupted whenever cells are passaged. Matrigel and decellularized ECM are two examples of materials used successfully for 3D cultures.[166,167] This culture technique supports long-term *in vitro* studies allowing cellular bonds that resemble those found in native tissue in terms of spatial distribution, and ECM can be synthesized over time without the problem of over-confluency. The transplantation of 3D cultures might also have a positive impact on cell survival after implantation since cell–cell connections and cell–ECM interactions are preserved in the structure. When Matrigel or decellularized ECM support 3D cultures, they provide a wide variety of complex ECM molecules to the culture. Artificial spider silk, on the other hand, merely provides a physical scaffold without any pre-existing ECM components present, which differs from the previously mentioned matrix structures.

In Paper IV, hfcMSCs were shown to readily attach to a scaffold composed of a new kind of artificial spider silk fiber, NT2RepCT. The cells formed connections to the NT2RepCT fibers through vinculin, indicating the formation of focal adhesions between the fibers and hfcMSCs.[168] The hfcMSCs formed an ECM containing collagen IV, laminins and perlecan, the essential components of basal membranes.[79] This culture method seemed promising as a means to provide support and in this way increase cell survival after transplantation. Unfortunately, we found that NT2RepCT, with or without hfcMSC 3D cultures attached, was not immunologically inert, as was reported to be the case for spider silk proteins in general.[95,169] Rejection was apparent at 4 weeks also in animals treated with costimulation blockade. Attempts to remove LPS through additional washing steps and production in bacteria with defective LPS (clear coli) did not decrease the immune response. When NT2RepCT scaffolds were transplanted alone, without hfcMSC attached to the fibers, we observed a more intense immune reaction than that directed to the transplanted 3D cultures, where hfcMSCs and their ECM components covered major parts of the fibers. From these findings, we concluded that bacterial residues or epitopes of NT2RepCT were the likely reason for the earlier immune rejection observed, as compared to hfcMSCs transplanted in Matrigel. The immune rejection visible around the transplanted 3D cultures at 4 weeks was similar regardless of whether the mice received costimulation blockade or isotype control antibodies. However, very few hfcMSCs survived in animals treated with isotype control antibodies, while costimulation blockade allowed for hfcMSC survival for 4 weeks but not 8 weeks. In previous studies, animals treated with isotype control antibodies very rarely displayed any surviving xenogeneic or allogeneic cells. In Paper IV, they were few in number, though present in three out of six animals, potentially due to the protective environment formed by the NT2RepCT scaffold covered by ECM components.

Transplanting a 3D structure with attached cells instead of injecting cells in single cell suspension may potentially enhance cell survival. Our results described in Paper IV do not contradict this notion, but do show that NT2RepCT is immunogenic, which overrides any beneficial effects. Other spider silk fibers have been reported to be immunologically inert.[91,92] The discrepancy of our results could potentially be due to the properties of the NT2RepCT protein, rendering it more immunogenic, or the immunogenicity of spider silk fibers in earlier studies failed to be recognized. Since stainings for lymphocytes have not been performed in previous studies of spider silk implants,[92,94] it is difficult to compare our results with earlier publications. However, other studies have shown macrophages surrounding the spider silk, which could be indicative of immunogenicity.[91,99] To elucidate whether it is a novel or altered epitope on NT2RepCT or perhaps bacterial residues that trigger the observed immune reaction, there are ongoing efforts to use human cells for NT2RepCT production instead of bacteria. In its current form, NT2RepCT cannot be used *in vivo*; however, it holds potential for improving *in vitro* studies where 3D cultures could be of benefit such as in, for example, toxicology studies.

Decellularized scaffolds or collagens may be better candidates for the delivery of cells and several studies have indicated their negligible immunogenicity.[166] However, it is reasonable to expect that the peptides contained in allogeneic or xenogeneic ECM components may be able to evoke an immune reaction. This expectation is further illustrated by the reactions observed to collagen fillers[170] and the role of collagens in autoimmune disease.[171] Therefore, the use of any 3D scaffold in transplantation has to take into account that additional antigens may complicate the treatment of immune rejection.

In conclusion, cell therapy remains a challenge and cell interaction with the new environment is crucial for successful engraftment. The cell selected, immunological processes involved and interactions with the components of the ECM will collectively affect the outcome. This thesis introduces a novel kind of MSC as a potential candidate for cell therapy targeting heart disease. Synergistic effects have also been demonstrated in terms of cell retention when anoikis and immune rejection are addressed simultaneously. Further knowledge about the interaction between cells and their ECM as well as the fundamental mechanisms controlling our immune system is needed for cellular therapy to serve as a future treatment option.

6 CONCLUSIONS

- Foxp3^{+} Tregs likely mediate immunologic acceptance induced by costimulation blockade treatment and are found close to engrafted xenogeneic or allogeneic cells.

- MSCs or Matrigel co-transplantation synergize with costimulation blockade treatment for the acceptance of allografts or xenografts in mice.

- hfcMSCs form a vessel-like structure expressing CD31, laminin α4 and fibronectin, indicating vasculogenic potential.

- The artificial spider silk NT2RepCT supports the 3D cell culture of hfcMSCs with the formation of a complex ECM.

- The NT2RepCT produced in bacteria is immunogenic *in vivo*.

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8 REFERENCES

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